From single compound to ambient air *in vitro* Exposure: enabling the toxicity assessment of allergy related air pollutants

Cumulative Dissertation

for the attainment of the academic degree
doctor rerum naturalium (Dr. rer. nat.)
from the Faculty of Mathematics and Natural Science
of the University of Rostock

submitted by
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Rostock, September 2024

https://doi.org/10.18453/rosdok_id00005125

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Year of submission: 2024

Year of defense: 2024

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Doktorandinnen/Doktoranden-Erklärung gemäß § 4 Absatz 1 Buchstaben g und h der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Rostock

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Ich habe eine Dissertation zum Thema:

„ From single compound to ambient air in vitro Exposure: enabling the toxicity assessment of allergy related air pollutants."

an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Rostock angefertigt. Dabei wurde ich von Herrn **Professor Ralf Zimmermann** betreut.

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Contribution to Peer-Reviewed Publications

First-authorships

The following manuscripts were contributed by Elias J. Zimmermann as first author and published in the peer-reviewed journals. The contribution of Elias J Zimmermann is declared below.

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1 **Title:** Biological impact of sequential exposures to allergens and ultrafine particles rich combustion aerosol on human bronchial epithelial BEAS-2B cells at the air liquid interface

Authors: Elias J. Zimmermann, Joana Candeias, Nadine Gawlitta, Christoph Bisig, Stephanie Binder, Jana Pantzke, Svenja Offer, Narges Rastak, Stefanie Bauer, Anja Huber, Evelyn Kuhn, Jeroen Buters, Thomas Gröger, Mathilde N. Delaval, Sebastian Oeder, Sebastiano Di Bucchianico, Ralf Zimmerman

Journal: Journal of Applied Toxicology *(Impact Factor: 3.6, 2021)*

Year: 2023

Elias J Zimmermann contributed to this study by being included in the design and implementation of the study. In short, the implementation and handling of the used culture system, the performance of all exposures, the performance of the metabolic activity assay and the RNA workflow for gene expression analysis. Moreover, he evaluated all published biological data, worked in the process of physicochemical data integration, as well as wrote and revised the manuscript.

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2 **Title:** Toxicological effects of long-term continuous exposure to ambient air on human bronchial epithelial Calu-3 cells exposed at the air-liquid interface

Authors: Elias J Zimmermann, Anusmita. Das, Anja Huber, Nadine Gawlitta, Evelyn Kuhn, Christoph Schlager, Bastian Gutmann, Tobias Krebs, Jürgen Schnelle-Kreis, Mathilde N. Delaval, Ralf Zimmermann

Journal: Environmental Research (Impact Factor 8.4, 2021)

Year: 2024 (Submitted)

Elias J Zimmermann contributed to this study by being included in the design and implementation of the study. In short, the performance of the cell exposures, and the RNA workflow for gene expression analysis. Moreover, he evaluated all published biological data, worked in the process of physicochemical data integration, as well as wrote and revised the manuscript.

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3 **Title:** Impact of Volatile and Semi-volatile Organic Compounds from Farming Environments on Allergy-Related Cellular Processes

Authors: Nadine Gawlitta, Elias J. Zimmermann, Jürgen Orasche, Anja Huber, Jeroen Buters, Sebastiano Di Bucchianico, Sebastian Oeder, Thomas Gröger, Ralf Zimmermann

Journal: Exposure and Health *(Impact Factor: 11.4, 2020)*

Year: 2021

Elias J Zimmermann contributed to this study by being included in the design and implementation of the biological parts in this study. In short, the performance of all exposures, the performance of the RNA workflow for gene expression analysis. Moreover, the evaluation and illustration of all published biological data, as well as helped to write and revised the manuscript.

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4 **Title:** The priming effect of diesel exhaust on native pollen exposure at the air-liquid interface

Authors: Joana Candeias, Elias J. Zimmermann, Christoph Bisig, Nadine Gawlitta, Sebastian Öder, Thomas Gröger, Ralf Zimmermann, Carsten B. Schmidt-Weber, Jeroen Buters

Journal: Environmental Research *(Impact Factor: 6.5, 2020)*

Year: 2022

Elias J Zimmermann contributed to this study by being included in the design and implementation of the experimental parts in this study. In short, the performance of all exposures, the production and observations of the aerosol generation and revised the manuscript

Related publications, not included in this thesis

5 **Title:** An alternative in vitro model considering cell-cell interactions in fiber-induced pulmonary fibrosis

Authors: Jana Pantzke, Svenja Offer, Elias J. Zimmermann, Evelyn Kuhn, Thorsten Streibel, Sebastian Oeder, Sebastiano Di Bucchianico, Ralf Zimmermann

Journal: Toxicology Mechanisms and Methods *(Impact Factor: 4.0, 2021)*

Year: 2022

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6 **Title:** Processing of carbon-reinforced construction materials releases PM2.5 inducing inflammation and (secondary) genotoxicity in human lung epithelial cells and fibroblasts

Authors: Jana Pantzke, Arne Koch, Elias J. Zimmermann, Narges Rastak, Svenja Offera, Christoph Bisig, Stefanie Bauer, Sebastian Oeder, Jürgen Orasche, Petra Fiala, Michael Stintz, Christopher P. Rüger, Thorsten Streibel, Sebastiano Di Bucchianico, Ralf Zimmermann

Journal: Journal of Hazardous Materials *(Impact Factor: 14.2, 2021)*

Year: 2022 (Under Review)

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7 **Title:** Effect of Atmospheric Aging on Soot Particle Toxicity in Lung Cell Models at the Air-Liquid Interface: Differential Toxicological Impacts of Biogenic and Anthropogenic Secondary Organic Aerosols (SOAs)

Authors: Svenja Offer, Elena Hartner, Sebastiano Di Bucchianico, Christoph Bisig, Stefanie Bauer, Jana Pantzke, Elias J Zimmermann, Xin Cao, Stephanie Binder, Evelyn Kuhn, Anja Huber, Seongho Jeong, Uwe Käfer, Patrick Martens, Arunas Mesceriakovas, Jan Bendl, Ramona Brejcha, Angela Buchholz, Daniella Gat, Thorsten Hohaus, Narges Rastak, Gert Jakobi, Markus Kalberer, Tamara Kanashova, Yue Hu, Christoph Ogris, Annalisa Marsico, Fabian Theis, Michal Pardo, Thomas Gröger, Sebastian Oeder, Jürgen Orasche, Andreas Paul, Till Ziehm, Zhi-Hui Zhang, Thomas Adam, Olli Sippula, Martin Sklorz, Jürgen Schnelle-Kreis, Hendryk Czech, Astrid Kiendler-Scharr, Yinon Rudich, Ralf Zimmermann

Journal: Environmental Health Perspectives *(Impact Factor: 11.0, 2021)*

Year: 2022

8 **Title:** Exposure to naphthalene and β-pinene-derived secondary organic aerosol induced divergent changes in transcript levels of BEAS-2B cells

Authors: Michal Pardo, Svenja Offer, Elena Hartner, Sebastiano Di Bucchianico, Christoph Bisig, Stefanie Bauer, Jana Pantzke , Elias J Zimmermann, Xin Cao, Stephanie Binder, Evelyn Kuhn, Anja Huber, Seongho Jeong, Uwe Käfer, Eric Schneider, Arunas Mesceriakovas, Jan Bendl, Ramona Brejcha, Angela Buchholz, Daniela Gat, Thorsten Hohaus, Narges Rastak, Erwin Karg, Gert Jakobi, Markus Kalberer, Tamara Kanashova, Yue Hu, Christoph Ogris, Annalisa Marsico, Fabian Theis, Tali Shalit, Thomas Gröger, Christopher P Rüger, Sebastian Oeder, Jürgen Orasche, Andreas Paul , Till Ziehm, Zhi-Hui Zhang, Thomas Adam, Olli Sippula, Martin Sklorz, Jürgen Schnelle-Kreis, Hendryk Czech, Astrid Kiendler-Scharr, Ralf Zimmermann, Yinon Rudich

Journal: Environment International *(Impact Factor: 12.3, 2021)*

Year: 2022

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9 **Title:** Atmospheric aging increases the cytotoxicity of bare soot particles in BEAS-2B lung cells

Authors: Michal Pardo, Hendryk Czech, Svenja Offer, Martin Sklorz, Sebastiano Di Bucchianico, Elena Hartner, Jana Pantzke, Evelyn Kuhn, Andreas Paul, Till Ziehm, Zhi-Hui Zhang, Gert Jakobi, Stefanie Bauer, Anja Huber, Elias J. Zimmermann, Narges Rastak, Stephanie Binder, Ramona Brejcha, Eric Schneider, Jürgen Orasche, Christopher P. Rüger, Thomas Gröger, Sebastian Oeder, Jürgen Schnelle-Kreis, Thorsten Hohaus, Markus Kalberer, Olli Sippula, Astrid Kiendler-Scharr, Ralf Zimmermann, Yinon Rudich

Journal: Aerosol Science and Technology *(Impact Factor: 4.8, 2021)*

Year: 2023

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10 **Title:** The Chemical Composition of Secondary Organic Aerosols Regulates Transcriptomic and Metabolomic Signaling in an Epithelial-Endothelial In Vitro Coculture

Authors: Svenja Offer, Sebastiano Di Bucchianico, Hendryk Czech, Michal Pardo, Jana Pantzke, Christoph Bisig, Eric Schneider, Stefanie Bauer, Elias J. Zimmermann, Sebastian Oeder, Elena Hartner, Thomas Gröger, Rasha Alsaleh, Christian Kersch, Till Ziehm, Thorsten Hohaus, Christopher P. Rüger, Simone Schmitz-Spanke, Jürgen Schnelle-Kreis, Martin Sklorz, Astrid Kiendler-Scharr, Yinon Rudich, Ralf Zimmermann

Journal: Particle and Fibre Toxicology *(Impact Factor: 7,2, 2023)*

Year: 2024 (Submitted)

11 **Title:** Generation, characterization, and toxicological assessment of reference ultrafine soot particles with different organic content for air-liquid interface exposures

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Journal: Science of The Total Environment *(Impact Factor: 8.2, 2023)*

Year: 2024

Acknowledgements

This work was conducted in the time between January 2018 and September 2024 in the group Comprehensive Molecular Analytics (CMA) in the framework of the Joint Mass Spectrometry Center (JMSC).

I would like to take this opportunity to express my sincere gratitude and appreciation to the people who have who supported me during my doctorate here in the working group. Without your help this doctorate would not have been possible.

First of all, I would like to express my special acknowledgements for Professor Dr. Ralf Zimmermann for his support and supervision during my time at CMA and giving me the opportunity to work in many great projects.

Special thanks also go to my supervisors, Dr. Mathilde Delaval and Dr. Sebastian Öder, who supported me in each step during my PhD and for providing support in demanding periods of the past years. Thank you so much for your patience and your commitment.

In addition, I would like to thank my colleagues in Munich and Rostock, who supported and accompanied me during my doctorate. I would especially like to thank Christoph Bisig, Lukas Schwalb, Svenja Offer, Johannes Becker, Jana Pantzke, Nadine Gawlitta, Ramona Brechja, Seongho Jeong, Elena Hartner, Anja Huber, Jan Bendl and Barbara Giocastro for their support in the lab but also for being friends and enjoying time outside of work.

Furthermore, I would like to thank the entire working group of the Joint Mass Spectrometry Center for the wonderful time, their support and everything we have been through together in the last few years.

I also would like to acknowledge all national and international cooperation partners and their input on several parts of this project.

I want to express my deep gratitude to my family and friends, for their support constant help, during these years.

Finally, I would like to thank Barbara for her endless support in the past years, for always helping and cheering me up when I needed it and for unconditionally believing in me, even when the next steps are completely into the unknown. Thank you!

Many thanks to all of you.

Zusammenfassung

Die Häufigkeit allergischer Krankheiten hat in den letzten sechs Jahrzehnten stetig zugenommen und betrifft derzeit etwa 20 % der Weltbevölkerung. Obwohl mehrere epidemiologische Studien die Beteiligung von Luftschadstoffen an der allergischen Sensibilisierung und Krankheitsverschlimmerung belegt haben, sind die Prozesse und involvierten Faktoren noch nicht bekannt. Bisherige mechanistische *In-vitro*-Studien bewerteten hauptsächlich einzelne Quellen und haben daher wichtige allergieverstärkende bzw. allergieschützende Wirkungen übersehen. Diese Dissertation ermöglichte und erforschte diese Faktoren und Mechanismen, die an der allergieverstärkenden bzw. der allergieschützenden Wirkungen beteiligt sind.

Zu diesem Zweck verwendeten wir verschiedene hochmoderne *In-vitro*-Expositionssysteme für die Zellexposition, in Kombination mit umfassenden physikalischen und chemisch analytischen Charakterisierungstechniken. In einer ersten Studie führte die zusätzliche Vorexposition menschlicher bronchialer Epithelzellen (BEAS-2B) mit Birkenpollenextrakt (BPE) oder Hausstaubmilbenextrakt (HDME) zu einer verstärkten zelluläre Reaktion verglichen zur einfachen Exposition mit UFP-reichem Verbrennungsaerosol. Eine einmalige Exposition mit UFP, BPE oder HDME führte zu Genotoxizität, und UFP induzierte zeitabhängig transkriptionelle Veränderungen, die mit proinflammatorischen und xenobiotischen Signalwegen verbunden sind. Eine zusätzliche Exposition mit Allergenen führte zu einer verstärkenden zellulären Reaktion, die sich durch eine ausgeprägtere und schnellere Modulation entzündungsfördernder und xenobiotischer Signalwege zeigte. Insbesondere die Aktivierung des AhR-Signal Weges erwies sich als wichtiges Merkmal der UFP-Toxizität und war involviert in der verstärkten zellulären Reaktion. Ähnliche Ergebnisse konnten beobachtet werden, wenn BEAS-2B-Zellen zuerst mit einem Diesel Aerosol exponiert wurden, bevor sie ganzen Birkenpollen ausgesetzt wurden. Bei zusätzlicher Exposition führte die zelluläre Reaktion auf Birkenpollen schneller und früher zur Expression von Asthma- und Entzündungs-bezogenen Genen.

Um die allergiebedingte Schutzwirkung und die mögliche Beteiligung kleiner organischer Verbindungen an dieser Schutzwirkung zu untersuchen, wurde die inhalierbare Feinstaub- und Gasphase einer allergieschützenden Umgebung (Kuhstall) und einer nicht schützenden Umgebung (Schafstalls), beprobt und charakterisiert. Es konnten vier verschiedene Verbindungen identifiziert, und in folgenden *In-vitro*-Zellexpositionen weiter untersucht werden. Die Analyse der differentiellen Genexpression von BEAS-2B zeigte ein deutliches Regulationsmuster von proinflammatorischen, xenobiotischen und allergischen Entzündungsgenen für das gesamten Stallextrakt, die kleine Fraktion (<3 kDa) und die vier identifizierten Verbindungen. Somit konnte die immunmodulatorische Wirkung kleiner organischer Verbindungen sowie der vier identifizierten Verbindungen aus einer bekannten schützenden Umgebung bestätigt werden.

Während dieser Dissertation wurde die Expositionsdosis und Expositionszeit als Einschränkungen von *In-vitro*-Expositionsstudien zur Bewertung realistischer Expositionsszenarien identifiziert. Daher führten wir eine Abschlussstudie durch, die auf einem *In-vitro*-ALI-Expositionssystem basierte, angepasst an Langzeitexposition von 72 Stunden. Zum ersten Mal wurden *In-vitro*-Zellen an der Luft-Flüssigkeitsschicht für 72 Stunden lang kontinuierlich mit Umgebungsluft exponiert, was nicht nur die Machbarkeit der Technik zeigte, sondern auch Toxizität sowie differentielle Genexpressionsmuster in menschlichen bronchialen epithelialen Calu-3-Zellen induzierte.

In dieser Dissertation haben wir gezeigt, wie wichtig es ist, verschiedene hochmoderne *In-vitro*-Expositionssysteme in Kombination mit einer umfassenden physikalisch-chemischen Charakterisierung zu verwenden, um allergieverstärkende- und allergieschützende Umgebungen zu testen, identifizieren und analysieren.

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Abstract

The prevalence of allergic diseases was constantly increasing in the last six decades and affects currently about 20% of the worldwide population. While several epidemiological studies demonstrated the involvement of airborne pollutants in influencing allergic sensitization and disease exacerbation, the processes and involved factors are still not understood. Mechanistic *in vitro* studies mainly evaluated single sources and thus could have miss important allergy-adjuvant or allergy-protective effects. This dissertation enables and investigates the factors and mechanisms involved in allergy adjuvant and allergy protective effects.

For this purpose, we used different state-of-the-art *in vitro* exposure systems were used for subsequent cell exposures, combined with comprehensive physical and analytical chemical characterization techniques. In the first study, pre-exposure of human bronchial epithelial BEAS-2B cells to birch pollen extract or house dust mite extract resulted in an adjuvant effect following UFP-rich combustion aerosol exposure. Single exposure to UFP, BPE or HDME resulted in genotoxicity, and UFP induced transcriptional changes linked to pro-inflammatory as well as xenobiotic related signaling pathways in a time dependent manner. Pre-exposure to allergens led to an adjuvant effect as indicated by a more pronounced and faster modulation of pro-inflammatory and xenobiotic signaling pathways. Activation of AhR signaling was especially shown to be an important feature of UFP toxicity as well as during the observed adjuvant effect. Similar findings could be observed by first priming BEAS-2B cells to a diesel model aerosol before exposure to whole native birch pollen. With additional exposure, the cellular response to birch pollen occurred faster and earlier expression of asthma and inflammation related genes.

To investigate the allergy related protective effect and the possible involvement of small organic compounds for this protective effect, the respirable PM and gas phase of a cow shed, a known protective environment, and a not protective environment, a sheep shed, was sampled and characterized. Four distinct compounds could be identified and were further evaluated with *in vitro* cell exposures. Differential gene expression analysis of BEAS-2B, indicated distinct regulation pattern of pro inflammatory-, xenobiotic- and allergic inflammation related genes, for the whole extract, the small fraction (<3kDa) and the four identified compounds. Thus, the immunomodulatory effect of small organic compounds as well as the four identified compounds from a known protective environment were confirmed.

In the course of this dissertation, the exposure dose and time were identified as limitations of *in vitro* exposure studies evaluating realistic exposure scenarios, and thus we conducted a final study, focused on a state-of-the-art *in vitro* ALI exposure system adapted to long term exposures for 72h. For the first time, *in vitro* cells were exposed continuously to ambient air for 72h at the ALI, resulting not only in the applicability of the technique, but also in induced toxicity and indicated gene expression patterns in human bronchial epithelial Calu-3 cells even with only ambient air exposure.

In this dissertation, we demonstrated the importance of using different state of the art *in vitro* exposure systems combined with comprehensive physicochemical characterization to evaluate allergy adjuvant- and allergy protective-environments.

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1. Motivation & Scope

Ambient outdoor and indoor air pollution is recognized as a leading public health problem worldwide by contributing to a variety of chronic lung diseases such as COPD (chronic obstructive pulmonary disease), allergic diseases and asthma, as well as cardiovascular diseases such as bronchiolitis heart diseases, strokes and cancer [2-4]. According to the World Health Organization (WHO), 99% of the world population live in places not fulfilling WHO air quality guidelines leading to approximately 7-8 million premature deaths each year [5, 6]. Air pollutants represent a complex mixture of biological, chemical and physical compounds including particulate matter (PM), organic compounds such as polycyclic aromatic hydrocarbons (PAHs) and trace gases such as carbon monoxide, sulfur dioxide, nitrogen dioxide and ozone [2] (**Figure 1**). These can be released by natural sources such as volcanic eruptions, wildfires, dust storms and plants [7] although anthropogenic activities contribute enormously to the total amount of air pollutants with increasing industry, mobility, transportation, agriculture and energy production [8-10].

During the three past decades, epidemiological and toxicological research has taught us a great deal about the adverse health effects of air pollutants [11-13]. Nevertheless, there are still large knowledge gaps in regard to their mechanism and influence on the development as well as exacerbation of severe diseases [14, 15]. The large complexity and interplay of different pollutants in the air [16], with steady emergence of newly produced materials raise significant concern for environmental- and human health and thus as well for regulatory agencies [17]. Specifically, the toxicological profile of a compound can be dramatically modified depending on single or co-exposures with other pollutants, potentially resulting in synergistic or antagonistic health effects [18, 19]. Not knowing the resulting effect poses great challenges and highlights the need for efficient, realistic, and physiological relevant *in vitro* exposure systems, considering single compound or mixtures, for investigating their toxicity and for deciphering their mechanisms of action and eventually allowing a reliable estimation of the human health risk. Such methods were successfully applied in this work for investigating the combined toxicological effect of various allergy-related and anthropogenic aerosols during short term exposure, the effect of long term exposure to ambient air, and highlighted the involvement of small chemical compounds in allergy protective environments. Moreover, this dissertation presents the relevance and adaptation of an automated exposure station (AES) for realistic long term *in vitro* exposure to ambient air.

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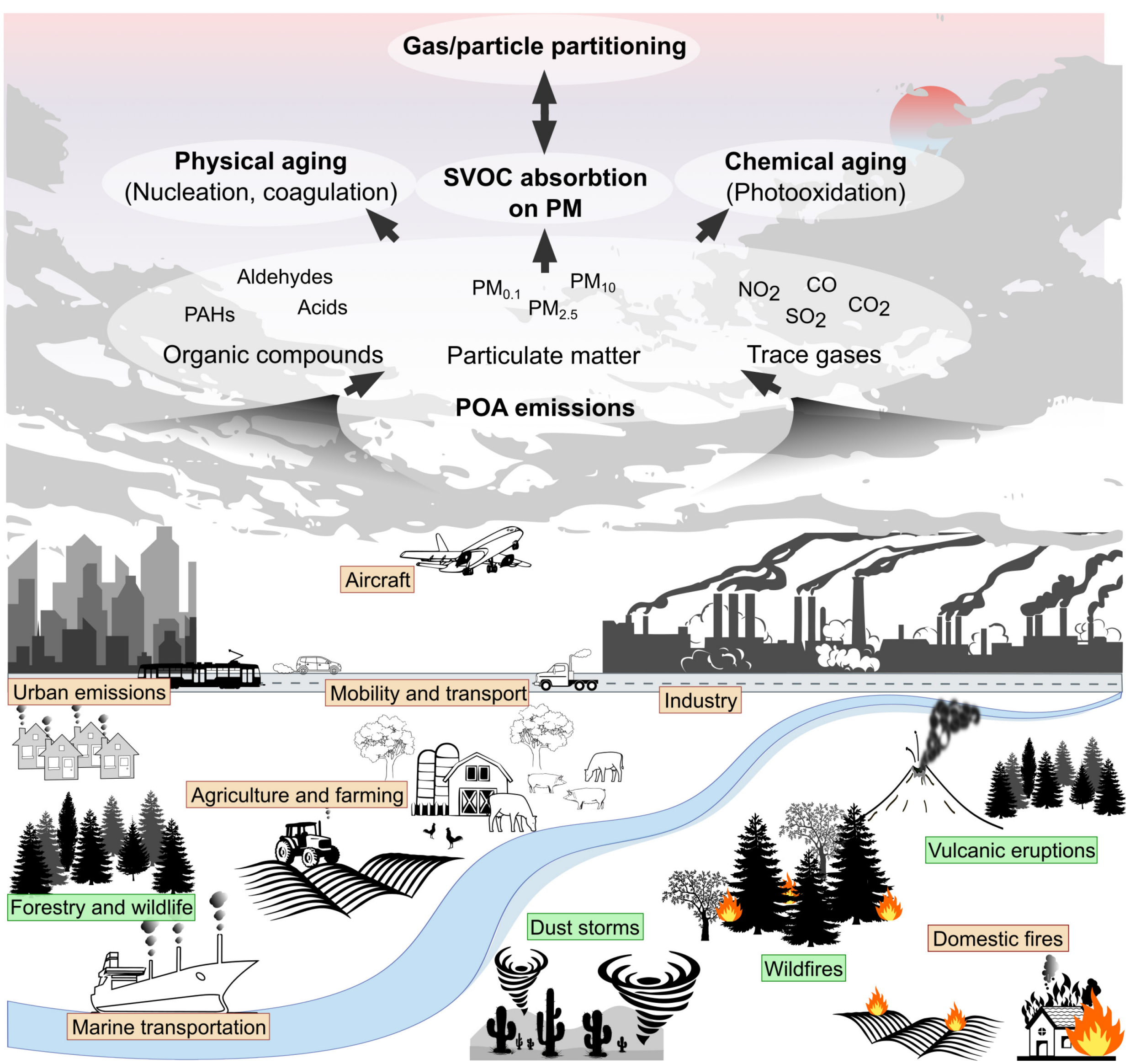


Figure 1: Sources of air pollution. Anthropogenic sources include emissions of aircraft, mobility and transport, marine transportation, agriculture and farming, industry, domestic fires, and urban emissions. Biogenic sources include forestry and wildlife, dust storms, wildfires, and volcanic eruptions. Both sources lead to direct primary organic aerosol emissions (POA) consisting of organic compounds like polyaromatic hydrocarbons (PAHs), aldehydes or acids, particulate matter (PM) in different size ranges as well as trace gases (NO_2, CO, CO_2, SO_2). POAs can undergo further physicochemical transformation by physical or chemical aging, alteration through absorption of semi volatile organic compounds on PM or transitioning between the gas/particle phase.

2. Introduction

2.1 Allergy

2.1.1 Prevalence of allergic diseases

The world is becoming allergic. In the last 6 decades started an increase in prevalence of autoimmune diseases, including allergic rhinitis, atopic eczema, food allergy and asthma mainly in high income countries [20]. But over the years also low-income countries began to have this increase in prevalence of allergic diseases [21, 22]. Although the disease prevalence of different allergic diseases started at different times in different countries [20] since their respective start, the increase is still ongoing reaching epidemic proportions affecting approximately 20% of the whole population worldwide [23]. This increasing disease prevalence is mainly attributable to global trends of increasing urbanization, changes in lifestyle (physical activity, diet, time spend indoors) and pollutant exposures [20-22, 24]. Moreover, there have been several studies on the relationship of these diseases, one leading to another, also known as the atopic march, indicating a common disease onset mechanism [25-28]. Despite the fact that a growing part of the population is affected by allergic diseases, patients with allergic diseases often perceive it as trivial and are thus is undertreated which can result in severe chronic upper airway diseases (SCUAD) [29, 30]. In general, all allergic diseases worsen the patient's quality of life, can limit daily activities, and can disrupt sleep during night [31]. To evaluate severity of allergic diseases questionnaires are performed including the Asthma Quality of Life Questionnaire (AQLQ), Saing George's Respiratory Questionnaire (SGRQ) or the Control of Allergic Rhinitis and Asthma Test (CARAT) [32, 33], which are considered respiratory disease specific. A more generalized approach is to determine the DALYs and years lived with disability (YLDs), were one Daly represent one lost year of healthy life, quantifying the burden of disease from morbidity and mortality together [31]. Especially the health impacts from asthma, the most extensive studied allergic disease [34], is dependent on disease severity and can reach from mild symptoms like coughing and sneezing to more severe symptoms including shortness of breath.

Besides the extensive impact of allergic diseases on human quality of life, the socioeconomic burden is quite extensive and needs to be considered as both direct and indirect costs. Direct costs are straightforward the resulting health care costs resulting from treatment but indirect cost are more debatable. Ernst [35] implemented a feasible approach where productivity loss is considered due to absenteeism as well as presenteeism, where inefficiency of labor is due to impairment.

2.1.2 Being and becoming allergic

Allergic (Immunoglobulin E (IgE)-associated) diseases are the most common immunologically mediated hypersensitivity disease and are based on the formation of IgE antibodies against usually harmless biogenic or anthropogenic antigens, termed allergens [36-38]. Depending on the site of allergic reaction, we can differentiate into inhalable allergies like allergic asthma or allergic rhinitis [23, 39], skin allergies [40], food allergies [41] and drug allergies [42]. Among these, asthma and allergic rhinitis are the

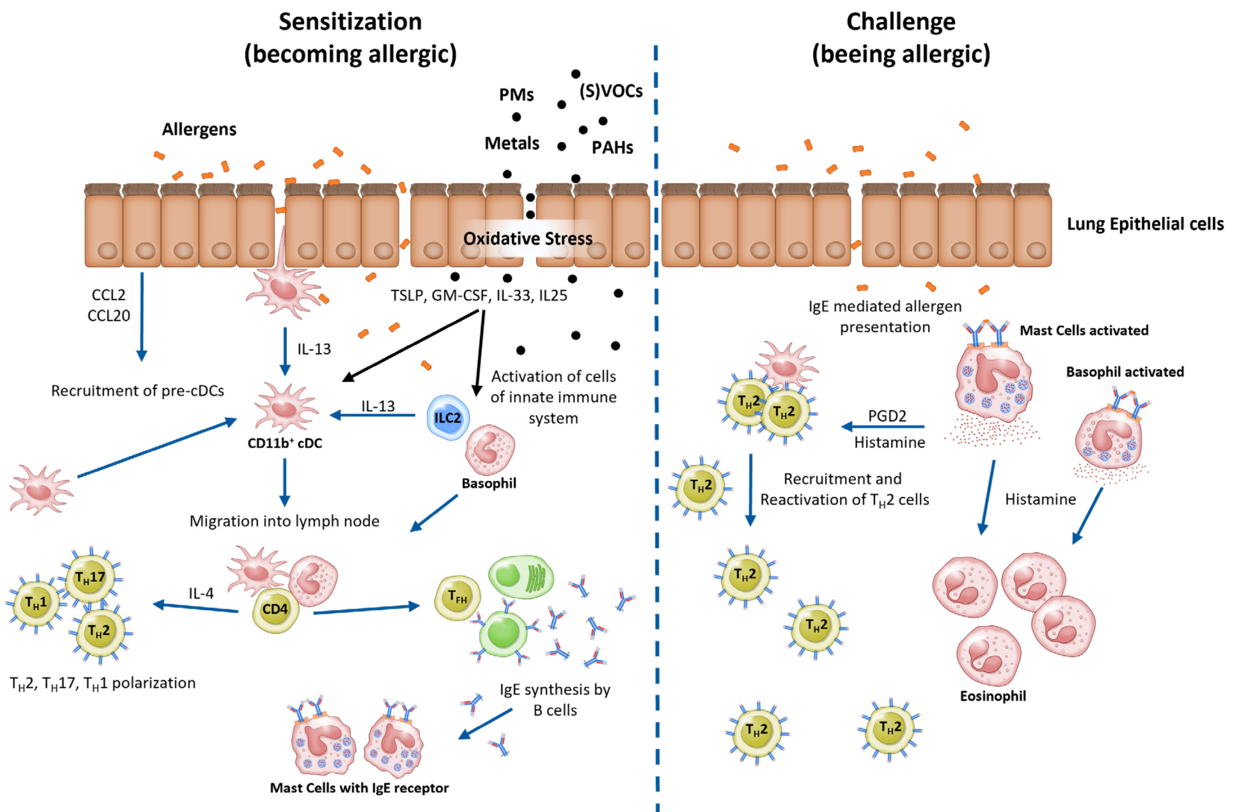


Figure 2: Schematic illustration of cellular mechanisms involved in (possibly) sensitization and allergic challenge of experimental asthma. Allergens can activate both epithelial cells and lung conventional dendritic cells (cDCs) through pattern-recognition receptors. Following activation, epithelial cells produce chemokines (CCL2 and CCL20) to recruit immature pre-cDCs. Air pollutants like particulate matter (PM), (semi-) volatile organic carbons (SVOCs) like polycyclic aromatic hydrocarbons (PAHs) and metals can damage bronchial epithelial cells leading to the production of reactive oxygen species (ROS) and thus oxidative stress. In response to air pollutants (including allergens), bronchial epithelial cells produce "instructive" cytokines (including TSLP, GM-CSF, IL-33 and IL-25) that initiate maturation of immature pre-cDCs into CD11b+ cDCs. These mature cDCs migrate with the help of IL-13, produced by ILC2, into lymph nodes and sustain with the help of basophils the TH2 response. In the lymph nodes, some T cells start producing IL-21 and adopt a follicular helper T cell (TFH) fate, inducing class switching to IgE in B lymphocytes (B cells). B cells start synthesis of IgE and released IgE primes mast cells. Throughout allergen challenge, CD11chi monocytic DCs and/or macrophages can locally restimulate resident lymphocytes and/or produce chemokines (CCL17/CCL22) to recruit effector TH2 cells. In humans, IgE-mediated allergen recognition enhances TH2 response to inhaled allergens. Adapted from [1]

most prevalent diseases with 1 to 20% and 1 to 18% in adults respectively, whereas the prevalence in children is considered to be even higher [31]. In the last few years, there have been many insights into the process of becoming allergic, but due to the complexity of the topic, it is still not understood. To current knowledge, becoming allergic starts at the first point of interaction between the host and the environment which is at the epithelial tissue. The epithelial tissue forms a continues layer that acts as first line of

defense against physical and chemical damage, allergens, infections, and environmental stress [43, 44]. Air pollutants like protease allergens, particulate matter (PMs), metals, (semi-) volatile organic carbons (SVOCs) including polycyclic aromatic hydrocarbons (PAHs) can either directly activate dendritic cells (DCs) or are first recognized by bronchial epithelial cells [45]. Activation of the bronchial epithelial cells can happen trough reactive oxygen species (ROS), leading to oxidative stress [46, 47], and by transcription activation trough pattern recognition receptors. In both cases production of cytokines like thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin 33 (IL-33) and interleukin 25 (IL-25) is initiated [45], which boosts DC activation and activates group 2 innate lymphoid cells (ILC2s)[48-50]. During this process the epithelial barrier takes damage, probably causing or at least aggravate the observed cytokines release and subsequent immune responses [45, 51]. Following activation, mature CD11b^{+} cDCs migrate into the lymph node and activate Th2 immunity including polarization of T helper 2 (T_H2) cells, IgE synthesis by B cells and priming mast cells (MCs) and basophil cells (BCs) with produced IgE receptors [36, 52]. This process is accelerated by interleukin 4 (IL-4) and

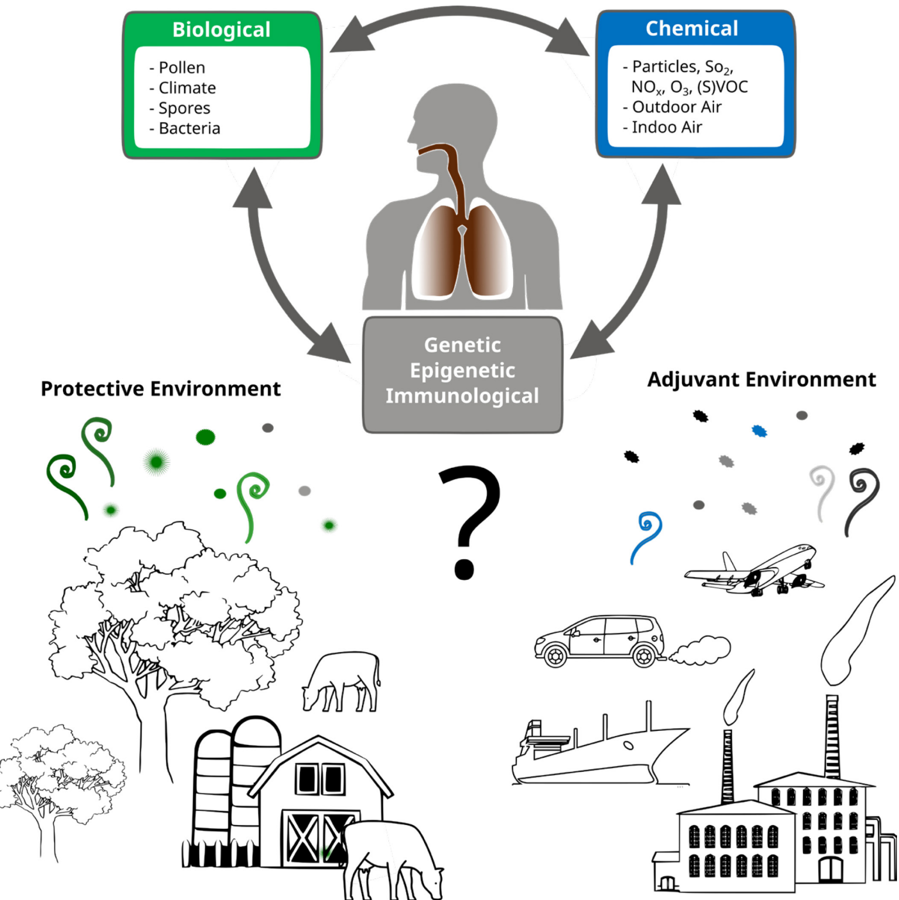


Figure 3: Schematic overview of factors influencing the onset of allergic diseases. Dependent on the environment the external effects can be protective (e.g. farm environments) or adjuvant (polluted, industrial environments) and influence together with intrinsic factors (genetic, epigenetic an immunological) the onset of allergic diseases, allergic disease exacerbations as well as each other.

interleukin 13 (IL-13) which is released by ILC2s and basophils that also migrated into the lymph node [53]. Currently it is hypothesized, that following many sensitization cycles to allergens and various other air pollutants, the epithelial barrier integrity decreases, the amount of T_H2 cells, DCs and IgE primed MCs/basophils drastically increase, leading to the hypersensitivity and overreaction to an allergen, also known as allergy [1, 39, 51, 54, 55].

2.2 Environmental impact on respiratory allergic diseases – adjuvant and protective effects

Given the fact that the allergic onset is influenced by a complex combination of many known and unknown factors [20], indoor- and outdoor- air (pollutants) are considered to be among the major influencing factors [56], but not always in a harmful way. Depending on the environment, we can differentiate between adjuvant and protective effects on the onset on allergic diseases (**Figure 3**). An adjuvant effect is given when allergic sensitization is stimulated and/or severity off allergic symptoms are increased. Airbourne compounds involved in this process can either be from natural sources, including pollen, spores, animal dander, wildfires, or volcanic eruptions but also from anthropogenic sources, including polluted indoor air, industrialization or traffic and transportation[45, 57, 58]. These compounds usually cannot directly induce symptoms of allergic diseases but influence the induction of the immunological pathways during allergen challenge, leading to a more severe response and thus promote sensitization [59]. In this regard, it has been shown that different compounds can trigger similar immunological

responses and dependent on their order lead to different immunological responses [60]. Especially traffic-related air pollution (TRAP), like PM from diesel exhaust particles (DEP), have been shown to have an adjuvant effect [22, 59-61]. Diesel is one of the most common used fuels and has been linked to reduced lung function, adjuvant effects in individuals with asthma and COPD, adjuvant effects in ischemic heart diseases and is also classified as carcinogenic [62-65]. DEPs consist of a small soot particle with, due it's small size < 2.5µm, a large surface area to which different compounds can bind. It does act as a carrier for various compounds including transition metals, PAHs, or aldehydes. PAHs include some known carcinogenic and mutagenic compounds, especially benzo[a]pyrene (BaP) [66], and transition metals can induce genotoxic effects [67]. In both cases, the generation of ROS is likely a key factor in their human allergenicity, as it damages the epithelial barrier and induces the release of immune cell recruiting cytokines. It has been demonstrated that DEP increased the allergy related Th2 response *in vitro* [68]as well as influenced the allergic inflammation *in vivo* [69, 70]. It is noteworthy to mention that adjuvant effects could also occur in an indirect way. Recent studies have demonstrated that with increasing temperatures and CO_2 levels, related to the climate change, not only the pollen season is extended but also that there is a substantial increase in pollen production from ragweed [71, 72] as well as significantly stronger allergenicity in pollen from birch trees grown at increased temperatures [73, 74]. In conclusion, this illustrates that beside the known airborne allergens, other airborne pollutants play an important role

in allergic immune reactions as well as the sensitization, though to what extend needs to be further investigated.

Since a few years it is also known that there are environments which can protect from becoming allergic. One of these environments is a cow shed, that is maintained in the classical way, without following high-tech industry in maintaining the shed. Children growing up on traditional farms were less susceptible to becoming allergic compared to children from non-framing environments [75, 76]. Due to their traditional farming approach, factors involved are the increased microbial diversity in their surroundings, contact with the animals and their feeding material like hay, the intake of unprocessed food like raw milk, their reduced time spend indoors as well as their contact to various compounds commonly found in traditional farming environments [75-79]. Especially the increased early life contact to endotoxins is hypothesized to be a major contributing factor to the observed decreased prevalence of allergic diseases [79, 80]. Some few experimental studies have been conducted and could confirm the observed epidemiological effect of protective farming environments in mice [80, 81]. Beside the involvement of microbial related endotoxin, non-microbial N-glycolylneuraminic acids were indicated to be protective for farmer children [82], as well as the concentration of butyrate and propionate in infants years correlated to the protective effects [83]. However, the underlying mechanisms of action of these compounds influence the onset of allergic diseases is not yet understood.

2.3 Relevance of combined airborne pollutants toxicity assessment

Given the fact that both adjuvant and protective effects on allergic disease onset are influenced by many different factors, investigating the knowledge gaps requires mechanistical studies evaluating the combined toxicological effects of airborne pollutants together with airborne allergens.

In this context, toxicological assessments need to have a representative dose/deposition which is rather complex within the human lung. Current lung deposition models describe the penetration and deposition of PM in various regions of the lung mainly dependent on the particle aerodynamic diameter [84] (**Figure 4 A**).

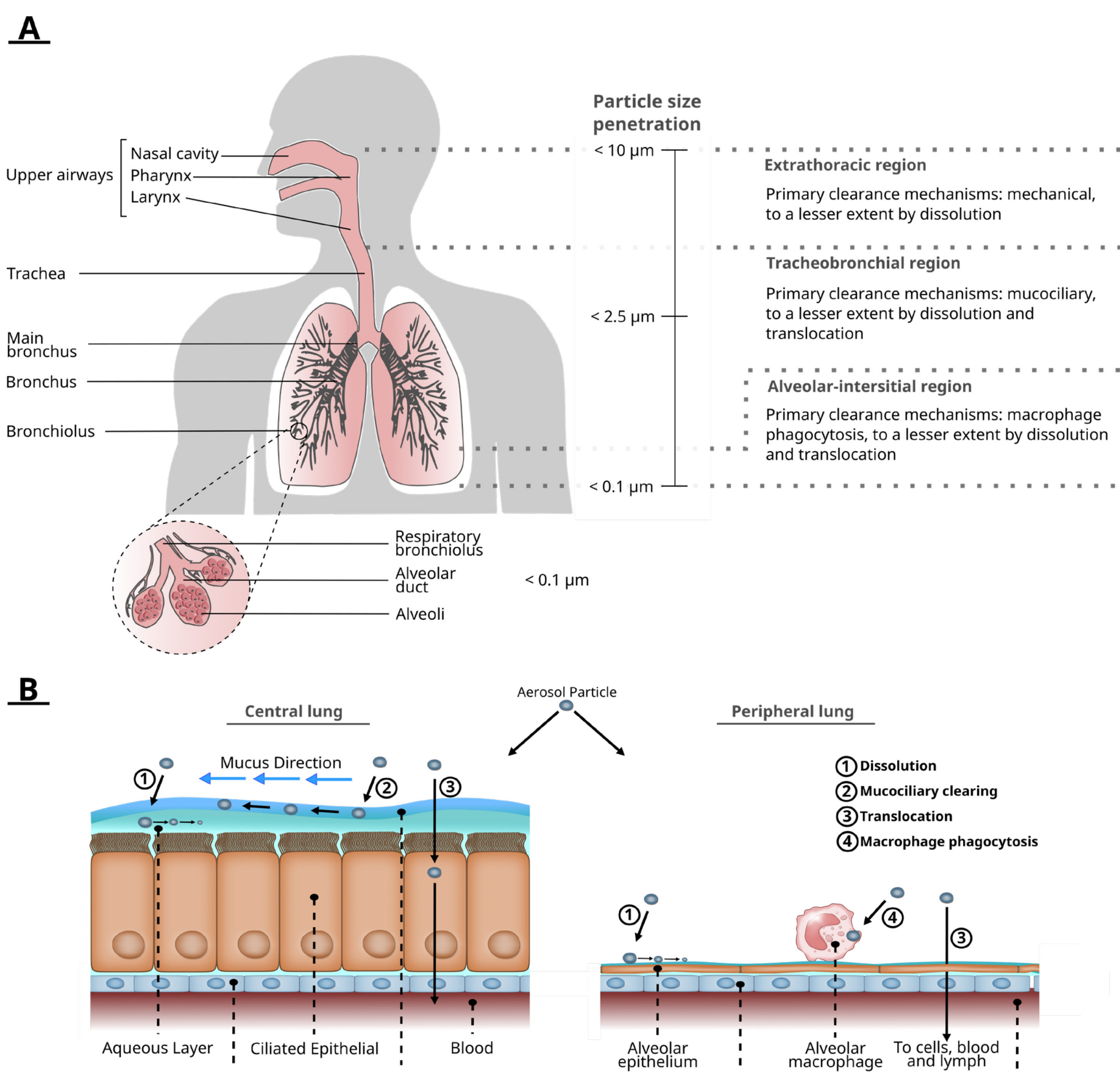


Figure 4, A: Schematic illustration of particle deposition in the human respiratory system in relation to size and clearance mechanisms. **B**: Particle clearing mechanisms in the central lung and the peripheral lung. Particles are either (1) dissolved, (2) transported away by mucociliary movement (only in the central lung), (3) translocated through the epithelial barrier reaching the systemic system, or (4) taken up and degraded by macrophages. Based on [85-87]

PM is generally categorized in relation to aerodynamic diameter: coarse PM (PM_{10}; particles with a diameter < 10 µm) fine PM ($PM_{2.5}$; particles with a diameter < 2.5 µm) and ultrafine PM (UFP; particles with a diameter < 0.1 µm) [88]. As already mentioned above, is deposition of inhaled aerosols a complex mixture that mainly depends on the size but also on other physicochemical properties of the particles, lung anatomy and respiratory pattern [89]. The main deposition mechanism in the respiratory tract of inhaled particles are impaction, sedimentation, and diffusion. Larger particles (< 10 µm) are mainly deposited by impaction due to inertia in the region of the upper airways (extrathoracic and

tracheobronchial region). Deposition by sedimentation occurs in regions of the lower airways for larger particles (< 2.5 µm) through the balance of gravitational vs drag force reducing the airflow velocity [89]. The 3rd mechanism is by diffusion and is the main deposition mechanism of the smallest particles (< 0.5 µm). Deposition by diffusion occurs mostly in the alveolar-interstitial region of the lung and is caused by Brownian motion causing collisions, due to longer residence time and air flow reduction, with the airway wall or air molecules [89].

Depending on the lung region and the physicochemical properties of the particles, the mechanisms of how these deposited particles are removed from the respiratory tract vary (**Figure 4 B**) [85]. Clearance of larger particles in the extrathoracic region is mechanically achieved through coughing, sneezing, or swallowing of the particles. In the tracheobronchial region, with its ciliated epithelium, insoluble particles are mainly removed by mucociliary clearance. By a coordinated ciliary movement, the particles are transported toward the larynx where they can be swallowed or removed as sputum. Dissolution by Macrophages is in the tracheobronchial region of minor importance but is the main clearance mechanism in the alveoli. Particles which cannot be removed by these mechanisms can be translocated from the tracheobronchial or alveolar region to other lung compartments as well as the blood circulatory or lymphatic system. [84, 89]. In this regard, especially UFPs can pass through the epithelial barrier and reach distant organs [90]. Moreover, due to their large surface area UFP can act as carriers of various (organic) material which can dissolve from the particles and thus be taken up by the epithelial cells [91]. Following deposition and possible translocation to distant organs, the inhaled pollutants can cause various local and systemic effects. These have been linked to the increase of various diseases including pulmonary diseases like COPD, asthma or lung cancer as well as cardiovascular disease like ischemic heart disease and stroke mortality [92-95]. The effects and severity of adverse health effects, pollutants cause is not only dependent on the particle inhaled but also on the combination of particles, as they directly influence each other. Especially UFPs, due to their large surface/volume ratio and physical properties can absorb compounds on their surface. This happens either through surface complexation and/or hydrogen hydrophobic, electrostatic, π-π stacking, covalent and/ or hydrogen bonding [96, 97]. The resulting complexes have a big impact on their distribution and fate within the organisms as well as the resulting biological effect [16]. **Figure 5** illustrates the mechanisms of joint toxicity of UFPs and co-pollutants which can either increase or decrease the severity of toxicity and pollutant concentration in the cells. Research on joint toxicity of air pollutants is scarce but increasingly illustrates their potential to promote or decrease disease entities in various organs induced by oxidative stress-related pathways and/or inflammatory stress-related pathways [16, 96-99].

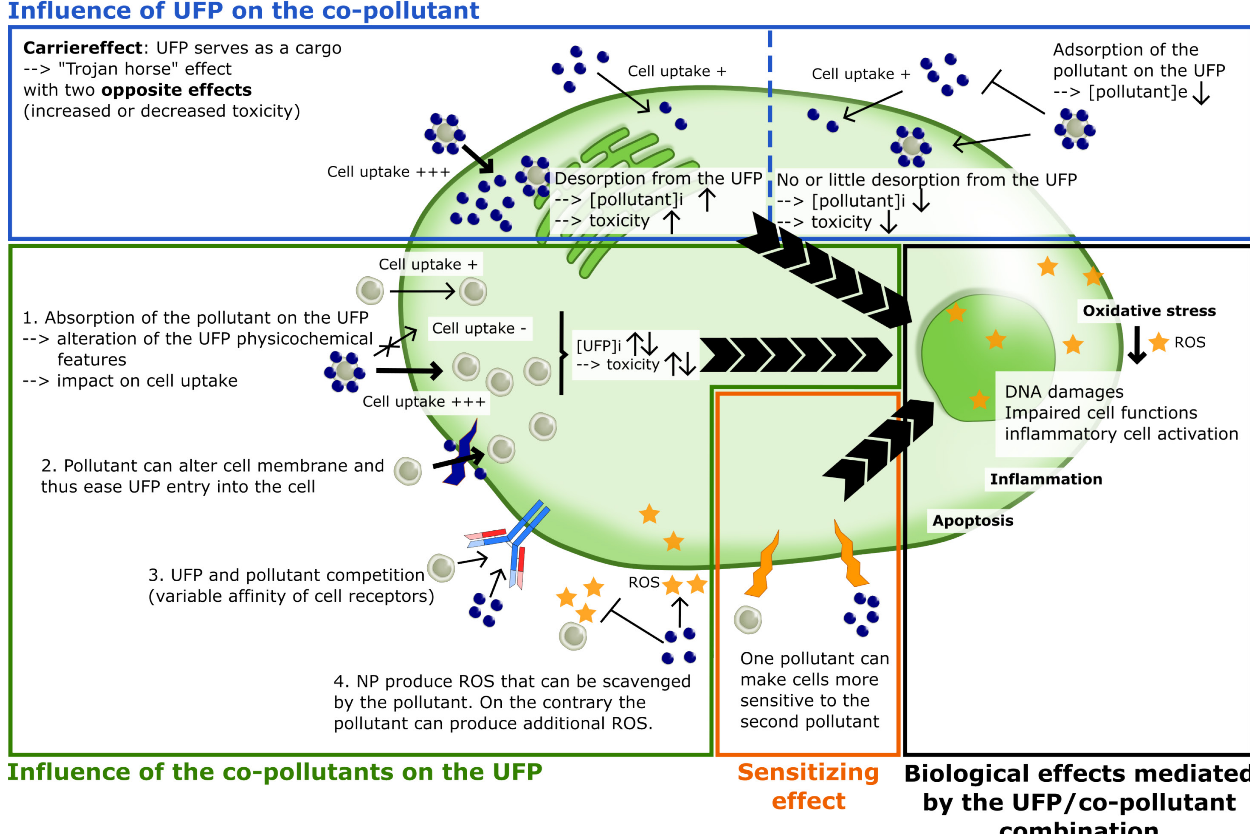


Figure 5: Schematic illustration of the potential mechanisms underlying the joined toxicity of UFP and co-pollutants. UFP: ultra fine particles; [pollutant]i: intracellular concentration of pollutant; [pollutant]e: extracellular concentrations of pollutant, [UFP]i: intracellular concentration of ultra fine particles; ROS: reactive oxygen species, ↓: decrease; ↑: increase. Based on [16]

2.4 Exposure systems to study airborne pollutants

The gold standard of testing airborne pollutants were so far *in vivo* exposure methods but there is a strong desire and a need to use alternative methods according to the 3R principle to reduce, refine or replace animal experiments [100]. *In vitro* models provide a powerful alternative evaluating the basic principle of pollutant induced toxicity, although they cannot reflect the comprehensive complexity of toxicological effects, detectable by *in vivo* methods [101]. In recent years, *in vitro* lung cell models cultured at the air-liquid interface have gained increasing interest for evaluating the toxicity of air pollutants. Indeed, they can be used in combination with aerosol exposure systems to mimic more closely realistic *in vivo* exposure conditions trough the preservation of the physicochemical characteristics of airborne particles [102] and the realistic size distribution comparable to particles deposited in the lung [103]. In the last few years, many different ALI exposure systems have developed, to evaluate the toxicity of PM as well as the gas phase. By including online aerosol measurement techniques, particle and gas phases can be adjusted and thus dose-related insights into toxic effects of various aerosols can be gained. Therefore,

they were already used for the evaluation of many pure emissions from exhaust engines, chemical compounds, trace gases and complex ambient air [104-106].

2.5 Scope of the work

Since the 60s, the prevalence of allergic diseases and allergic asthma is constantly increasing but the reasons for the increase and the underlying mechanisms of allergic disease onset are not yet understood. The onset of allergic diseases is very complex, and many factors are considered to be of great importance like indoor and outdoor aerosols. While many efforts flow into the scientific evaluation of single compounds of aerosols and their role in allergic disease development, aerosols are complex mixtures, consisting of thousands different biogenic and anthropogenic compounds, suggesting the importance of joint toxicity assessments and a comprehensive physicochemical characterization of relevant allergy-adjuvant and -protective environments. To gain further insights in the relationship of aerosols and allergic diseases, subsequent *in vitro* exposures in realistic exposure systems are needed coupled to sophisticated analytical techniques.

Therefore, the aim of this PhD thesis was to adapt and utilize different *in vitro* ALI exposure systems to gain a better understanding of allergy-adjuvant and -protective environments and the cellular and molecular response they induce. A focus was put on evaluating the physical parameters as well as the chemical composition of the tested aerosols to identify their importance in inducing cellular responses in in vitro human lung cells.

2.5.1 Specific research questions of the thesis

Subsequent exposures – adjuvant effects

- Do we observe an adjuvant effect after pollen- /house dust mite-extract pre-exposure and subsequent UFP exposure?
- Do we see an adjuvant effect after diesel emissions exposure and subsequent whole birch pollen exposure?
- Can we correlate specific compounds to the observed biological outcomes?

Collected ambient air exposures – protective effects

- Is there a difference in chemical composition of gaseous and condensed VOCs and SVOC between potentially protective and not protective farming environments?
- Can we see different immune modulatory effects between the total extract and the small organic compounds following in vitro human lung cell exposure?

Continued long term ambient air exposure

- Is it possible to expose in vitro human lung cells for 72h continues without observing effects due to the exposure?
- Are there different toxicological effects in cells exposed to ambient air and clean air?
- Are there different toxicological effects in cells exposed to ambient air for 72h during the weekend, the workweek, and the workweek with a holiday on the second day?

3. Methods

The following chapter gives a summary of the applied methods in this thesis. More information can be found in the publications from this thesis.

3.1 Aerosol Sampling, Production and Characterization

There are mainly two ways of how airborne particles from environments can be tested, namely sampling of an existing environment or by reproduction a specific condition in a reproducible manner. The needed time for adequate testing depends not only on the research question but also the environment to be tested. For our study in different shed environments, we used filter sampling on quartz fiber filter (QFF) in combination with a high-volume digital sampler with a pre-impactor to remove particles larger than 2.5 µm. The airborne particles were sampled 7 days in a row for 24 h with a high volume to adjust for the low particle concentration in and outside of the sheds (**Figure 6**). For the adjuvant effect studies of this thesis, the aerosol was directly produced by a miniCAST [107] or a dieselCAST, which mimics the physical and chemical properties of real fuel emission [108]. Both systems work with a diffusion flame to reproducible produce both gas- and particle phase. The sampling period for these experiments were set to 2 h as the concentration of generated particles was higher than what is observed in real environments. To guarantee reproducibility and comprehensive characterization of the aerosol, particle size distribution, particle mass, elemental- and organic carbon content, as well as SVOCs were identified, according to established methodology in our research group.

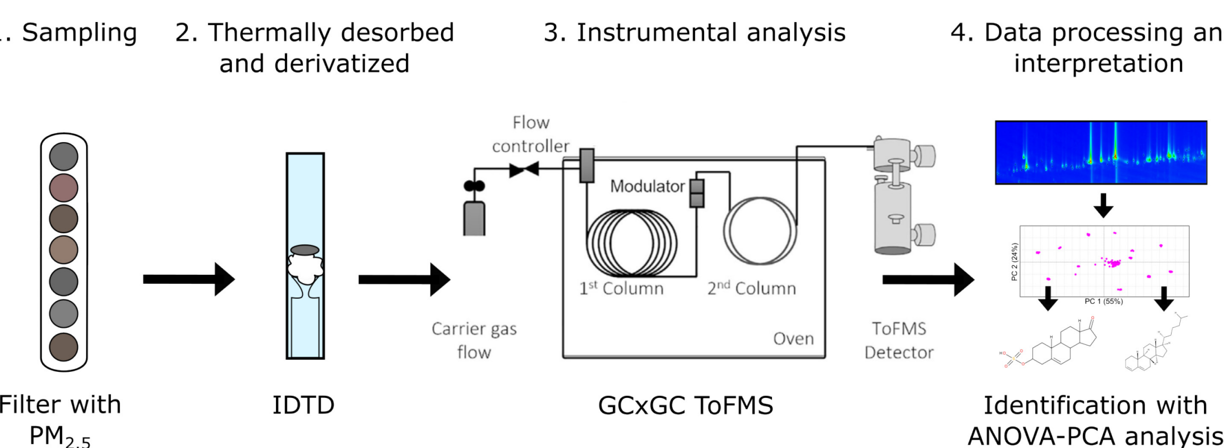


Figure 6: Schematic Workflow of chemical fingerprinting of protective (cow) and non-protective shed environments. 1. A high volume sampler was used to collect PM2.5 on quartz fiber filter (QFF). 2. Define cuts of filter were thermally desorbed and derivatized by in-situ derivatization thermal desorption (IDTD) approach. 3. GC×GC-ToFMS analysis. 4. Comprehensive characterization of PM2.5 with subsequent identification of key components by ANOVA-PCA analysis

3.2 *In vitro* exposures of human lung cells

3.2.1 Cell culture

In pulmonary research, *in vitro* cell cultures enable the possibility to establish complex, physiological relevant and cost-effective test systems. For ALI cultivation, cells were seeded on transferrable 24-mm Transwell inserts with a polyester membrane of 0.4 µm pore-size. After reaching targeted confluency, the apical media is removed, putting the cells at the ALI. This allows the cells to have contact with the culture medium on the basal side, while having on the apical side contact with air and thus representing a more realistic scenario compared to submerged cultivation [109]. Following an acclimatization phase to the ALI condition the *in vitro* model system can be used for the various exposure and subsequent evaluation of induced toxicity of the tested aerosol. For *in vitro* cell cultures, one can distinguish between primary cells lines and immortalized cell lines based model systems, with both advantages and disadvantages. Primary based material reflects more closely the realistic *in vivo* condition, although the donor variability is high and the availability is limited, making the repeatability hard. Immortalized cell lines are not as representative of the *in vivo* condition but allow the investigation of universal basic principles due to their repeatability as well as their constant availability [109]. Moreover, the gap to a more realistic model system can be achieved by making functional 3D models of different immortalized cell-lines [110]. To investigate the induced effect of airborne-compounds and -pollutants

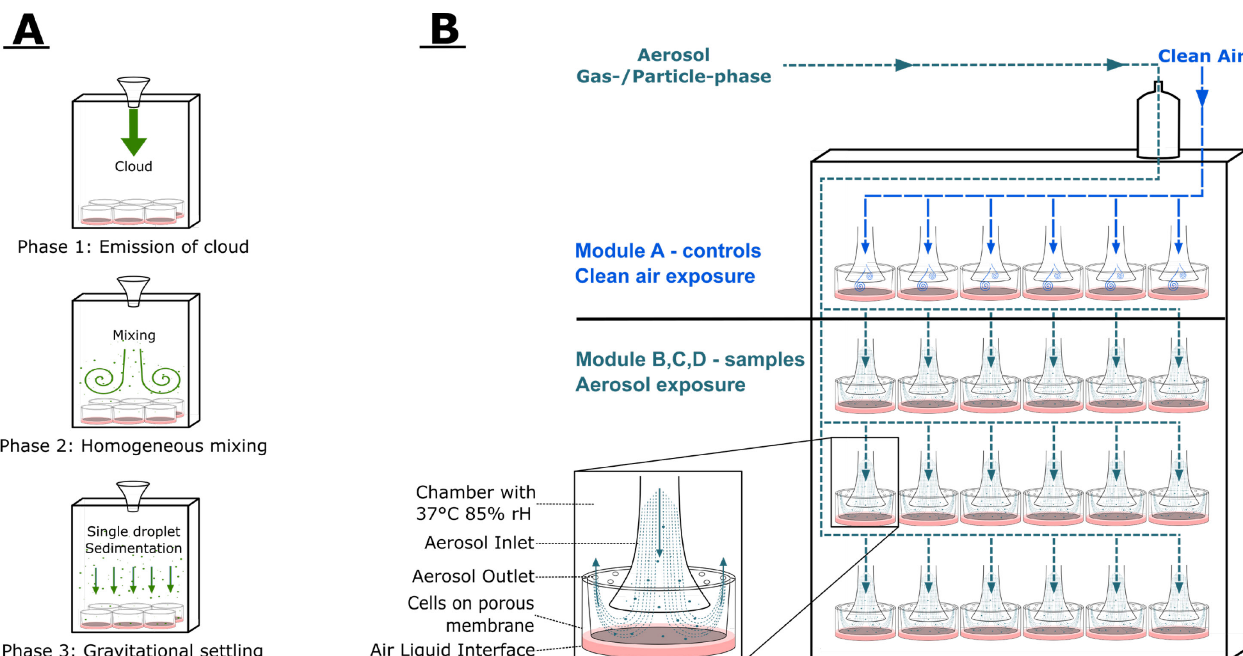


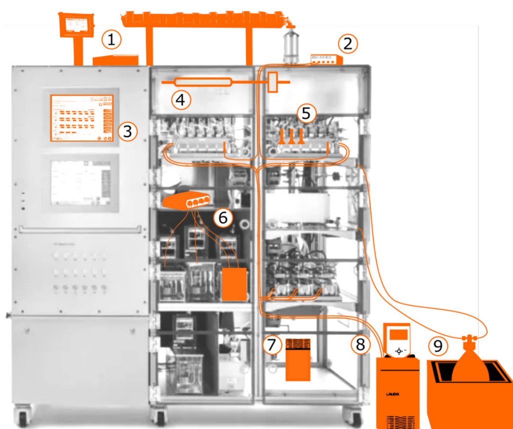
Figure 7: Principle of the Vitrocell® CLOUD system and the Vitrocell® automated exposure systems. (**A**) In the CLOUD system the particles of interest are added as solution to a nebulizer and the deposition is based on sedimentation. (**B**) In the automated exposure station (AES) the particle- and gas-phase are constantly flowing over the cells, resulting in sedimentation and diffusion dependent on the particle size and air flow over the cells

two *in vitro* lung cell culture models were used in this study. The non-tumorigenic human bronchial epithelial cell line BEAS-2B and the tumorigenic human bronchial epithelial cell line Calu-3. Both were chosen in regard to experimental setup and research questions. BEAS-2B cells have the big advantage of being non-tumorigenic, retaining many cellular processes and they are an established and widely used test system. Calu-3 cells were used during the long-term exposures, as they form tight junctions at the ALI, remain a monolayer, are able to withstand the airflow of continuous exposure systems and can be cultured for weeks at the ALI [111]. Both were cultivated according to the recommended ATCC guidelines to be comparable to similar (future) studies.

3.2.2 Air liquid interface (ALI) exposures

There are currently many different ALI exposure systems available for different use cases. They mainly vary in their mode of aerosol application as well as in the amount of knowledge the user needs for exposure and maintenance of the system. Due to their high specificity to certain aerosols, a combination of these systems allows for wider applicability and thus evaluation of more diverse research questions. This approach has been used in this thesis for both adjuvant effect studies where, in the first study, the cells were first exposed with the Vitrocell® Cloud chamber to birch pollen extract (BPE) or house dust mite extract (HDME) in solution before using the automated exposure system (AES) coupled to organic UFP production from the miniCAST. The Cloud chamber is a fast and easy to use exposure system, where the aerosol of interest is brought into solution and added to nebulizer. The nebulizer mechanically nebulizes the solution as a cloud into the chamber containing the cells. **Figure 6 A** illustrates the three main steps occurring during exposure. After the cloud is introduced into the chamber as 2-10 µm droplets, the droplets are homogeneously mixed in the chamber through occurring vortexes of the aerosol. This process takes place for about 1 min before the droplets sediment as single droplets onto the cells and homogeneously expose the cells [112]. The AES of Vitrocell®, used in the first study for generated organic UFP exposure, exposes the cells to a humidified laminar flow of particle- and gas-phase and needs thus to be directly connected to the source of interest. **Figure 6 B** illustrates the principles of the system and a detailed schematic of a single exposure position. The aerosol is perpendicular guided onto the cells at the ALI through a trumpet and out again through holes, set in a ring shape around the trumpet. To maintain a constant aerosol flow, a pump is used for the overall system air flow and each position has its own mass flow controller and sensor to control and maintain the flow. This is especially of importance, as the deposition efficiency is affected by the air flow conditions as well as the trumpet geometry and aerosol characteristics. In the second adjuvant study of this thesis the pre-exposure was again performed with an AES, tough this time to diesel exhaust, and subsequently to whole birch pollen in the pollen tower. The pollen tower provides the possibility to directly expose native/dry pollen to the cells at realistic doses and is based on the principle of sedimentation. One of the main limitations of *in vitro* exposures, on the way to replace *in vivo* exposures, were the fact that only acute toxicological effects could be investigated. Besides the possibility to use repeated exposures with relatively high doses there were no possibilities to

expose *in vitro* cells continuously to low dose of indoor- and outdoor-air. Therefore, the most advanced AES from Vitrocell® was further optimized to allow for continuous 72 h exposures. The modification of the systems is shown in **figure 7** and are mainly focused on a stable relative humidity (rH) and temperature at the cellular stages. Additional sensor for rH and temperature, a heated inlet before the system and more heaters and radiators were installed, to allow for more precise and stable control. Additionally, the user



1. Heated inlet
2. Modular r.H./T sensors
3. Software optimization
4. Adjustment humidification
5. Optimized Trumpet design
6. Automated refill system
7. Improved heater concept
8. Refrigerated circulation bath
9. Additional water trap

Figure 8: Optimization made to the Vitrocell® AES to enable continues long term ambient air exposures.

maintenance was reduced by using an automated refill system for both water baths needed by the humidification units as well as a secure remote access to the system was developed.

3.3 Toxicological evaluation of aerosol induced effects

Following the various exposures, the treated cells are either harvested directly or after a 2-22h post incubation period to evaluate additionally slowly induced cellular effects. For the toxicological evaluation a comprehensive cytotoxicity assessment was always first performed, to observe the degree of cytotoxicity and metabolic activity and to avoid data misinterpretation of subsequent in-depth analysis, caused by high cytotoxicity or disintegration of cellular products. Several tests were used in this thesis for the first and in-depth evaluation of the effects caused by the aerosol of interest and are further described below.

3.3.1 Metabolic activity

For measuring metabolic activity and assess cell viability, the resazurin reduction assay was used, also known as the alamar blue assay (**Figure 8**) [113]. This assay is based on the fluorometric redox reaction of the non-fluorescent dye resazurin to the strongly fluorescent dye resorufin. Living cells are metabolic active and take up the cell permeable dye resazurin before intracellular reduction with enzymes, like nicotinamide adenine dinucleotide (NADH) in the mitochondria, cytosol and microsome, to the also cell permeable dye resorufin. The released amount of resorufin is monitored and quantified by a

spectrophotometric microtiter well plate reader. This intracellular reduction is dependent on metabolic active cells and can thus directly correlated to the viability of the tested cells.

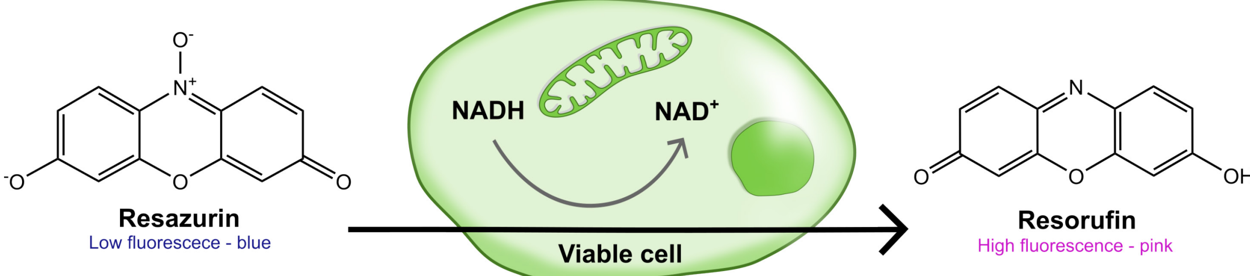


Figure 9:Basics of the resazurin assay. Resazurin, non-fluorescent and blue, is directly taken up by viable cells and metabolized by reduction of several redox enzymes, like nicotinamide adenine dinucleotide (NADH) in the mitochondria, cytosol, and microsome, to resorufin. Resorufin, highly fluorescence and pink, diffuses from the live cells back into the surrounding medium and can be detected. The detected fluorescence is direct proportional to the amount of metabolic activity of living cells.

3.3.2 Cytotoxicity

Lactate dehydrogenase (LDH) is a widely distributed, intracellular housekeeping enzyme that can be found in almost all living cells in high levels, as it is used for energy generation of glucose. It is a stable enzyme that is released into the surrounding medium upon damage and rupture of the cell membrane [114]. The LDH assay detecting the amount of LDH in the surrounding culture media is based on an enzymatic coupling reaction as shown in **Figure 9** [114]. Lactate is converted to pyruvate with the catalytic activity of LDH, producing the nicotinamide adenine dinucleotide (NADH) from NAD+. In a subsequent reaction NADH is consumed in a reaction from iodonitrotetrazolium to formazan. Formazan is a red dye with an absorbance at 490 nm, which can be quantified and is proportional to the amount of released LDH to the surrounding culture medium. By performing a positive control with a membrane lysing agent like TritonX, the maximum amount of released LDH can be evaluated and used to calculate the percentage of cytotoxicity in the samples of interest.

3.3.3 Life-dead cell imaging

Besides LDH release and metabolic activity evaluation, fluorescence microscopy is a fast and invaluable tool to get further insights into various cellular processes, including cell physiology as well as amount of damaged or dead cells. To get first insights into the integrity of the monolayer of the cells at ALI, simple and fast live-cell images were generated, with the membrane permeable Hoechst 33342

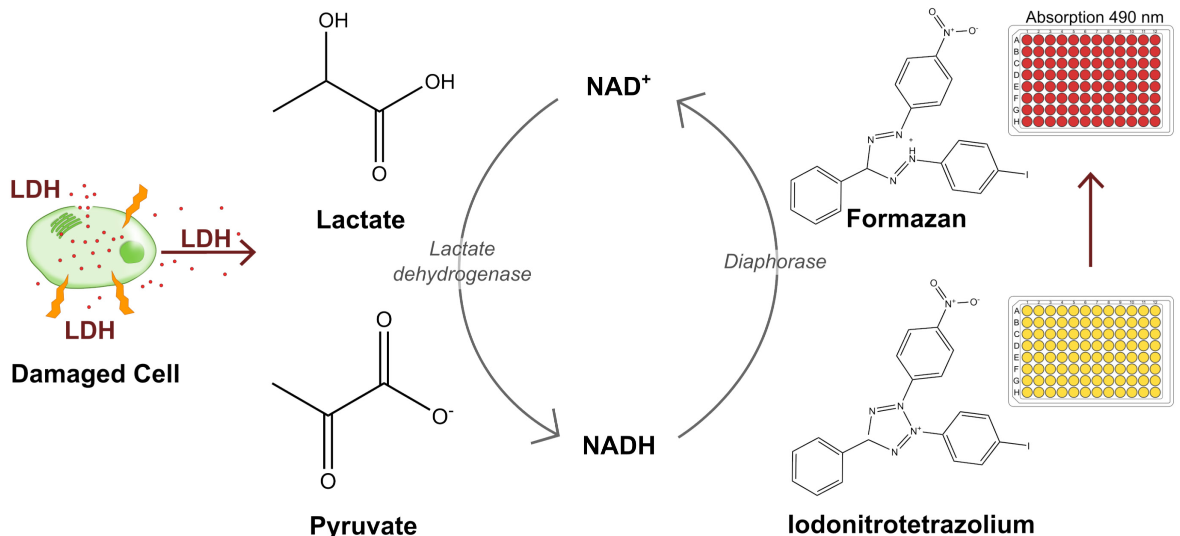


Figure 10: Basics of lactate dehydrogenase (LDH) assay. LDH is present in high levels in cells that uses glucose for energy and gets released to the surrounding medium when the cell membrane is damaged. In the medium LDH oxidizes lactate to pyruvate which induce the formation of iodonitrotetrazolium to formazan through the production of nicotinamide adenine dinucleotide (NADH). The amount of formazan can be detected through its absorption at 490 nm and is proportional to cytotoxicity

(minor groove DNA dye) to stain intact cells and the membrane impermeable propidium iodide (DNA intercalating dye) to stain the damaged and dead cells [115].

3.3.4 Immunostaining

To get an additional counterstain, 4`,6-diamidino-2-phenylindole (DAPI), a DNA intercalating dye, was used to stain all cells independent on their state. Additional to the live-cell staining's in Calu-3 cells, epithelial barrier integrity was evaluated with an immunostaining targeting tight junctions. Following exposure, the cells were first fixed on the membrane with paraformaldehyde, permeabilized with TritonX and stained with a ZO-1 monoclonal antibody as well as the DAPI counterstain [116].

3.3.5 Epithelial barrier integrity

Epithelial barriers are the first point of contact between the body and its surrounding environment, and the perturbation of the epithelial barrier integrity can have pathophysiological consequences, including allergic rhinitis [117]. Calu-3 cells cultured at the ALI produce an airway epithelial morphology and electrical resistance (including among others tight junction expression, secretory components production or have an active cystic fibrosis transmembrane conductance regulator) similar to *in vivo* conditions [116, 118]. Thus, to further evaluate the impact on continuous ambient air exposure on epithelial barrier integrity, transepithelial electrical resistance (TEER) measurements were performed, additional to the ZO-1 immunostaining. To perform these measurements, pre-warmed HBSS was shortly added to the apical and basolateral side of the TW.

3.3.6 Genotoxicity

Hazardous compounds can induce various harmful cellular effects, including genotoxicity by damaging the information on at DNA or chromosomal level. To detect single and double strand breaks the alkaline mini-gel version of the comet assay, also known as the single-cell gel electrophoresis assay [119], was used in the subsequent exposure study to allergen extracts and UFP. In principle, the cells are detached from the TWs and single cells are embedded in agarose mini gels on microscopy slides. On the slides follows a hypertonic salt solution lysis treatment to remove the cellular membrane and plasma components, the supercoiled DNA is un-wind by an alkaline treatment and the DNA fragments are separated with electrophoresis. During electrophoresis, intact DNA does not move towards the anode but DNA pieces, formed through single and double DNA strand breaks, move in the electrical field in relation to their size. This forms in the end a small head of undamaged DNA and a Tail consisting of the broken pieces, resembling a comet. Following staining, the samples can be microscopically evaluated, by comparing the size of the tail (broken DNA fragments) to the head (intact DNA) and calculating the percentage of DNA strand breaks.

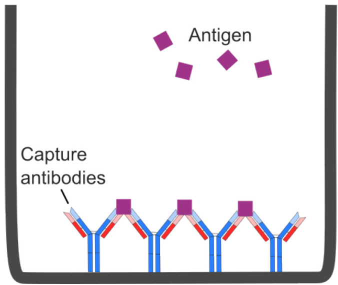
3.3.7 Reactive oxygen species indicator malondialdehyde

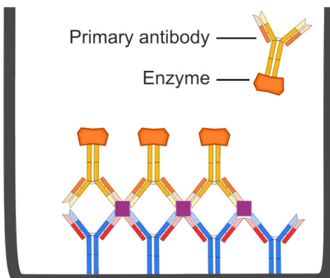
Within a biological system there is a redox homeostasis, a balance between redux, oxidative, and reducing reactions, and its disruption results in accumulation of oxidizing molecules, either through over production or loss of reduction ability [120]. ROS are produced by a partial reduction of oxygen molecules to superoxide, making them highly reactive, and able to react with any cellular building blocks. This includes carbohydrate, proteins, nucleic acids and lipids, whereas the reaction with lipids are known as lipid peroxidation [121]. During lipid peroxidation, polyunsaturated fatty acids are damaged, and produce in a relatively constant proportion malondialdehyde (MDA). This makes the quantification of MDA to a good indicator of lipid peroxidation and thus a indicator for ROS [122]. To quantify the amount of released MDA to the surrounding media, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used [123, 124]. Due to its high reactivity, MDA was first derivatized with 2,4-dinitrophenylhydrazine (DNPH) before quantification with LC-MS/MS.

3.3.8 Inflammatory cytokine quantification

During toxicological assessments, one of the first cellular responses is the induction and release of cytokines, corresponding to the encountered situations. In our study, we focused on pro-inflammatory cytokines interleukin 6 (IL-6) and interleukin 8 (IL-8) as one of the first inflammatory mediators of tissue damage [125]. To detect the release of IL-6 and IL-8 into the surrounding media, we used an approach based on the principle of a sandwich enzyme linked immunosorbent assay (ELISA) [126, 127]. **Figure 10** depicts the basic mechanisms of the sandwich ELISA. The assay starts by coating a 96-well plate with the capture antibody before adding the sample, containing the antibody of interest. Following some washing steps, the primary antibody with a bound enzyme is added and binds to the antigen of interest. The bound enzyme is a streptavidin-horseradish peroxidase conjugate, which can promote in the last step the color

reaction from the chromogenic (TMB) substrate to a colored product, which is quantifiable with a plate reader. The amount of colored product is dependent on the antigen of interest and can thus be quantified by using a standard curve.





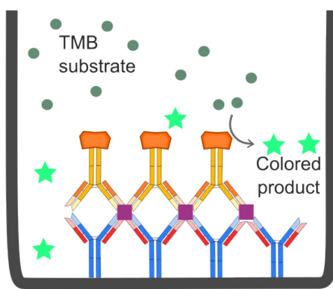


Figure 11: Basics of the sandwich enzyme linked immunosorbent assay (ELISA). In a 96 well plate the capture antibody is first bound to the solid phase and incubated with the target antigen which binds to the capture antibody. After washing, the primary antibody with bound enzyme (streptavidin-horseradish peroxidase conjugate) is added and binds to the antigen. This enzyme promotes the color reaction from the TMB (chromogenic) substrate which can be detected and is proportional to the amount of targeted antigen in the sample of interest.

3.3.9 Differential gene expression analysis

Above-mentioned techniques allow the assessment of a specific cellular response to a potential hazardous situation, but only roughly indicate the involved cellular mechanisms. To study the toxicological effect and the underlying mechanisms in more detail, In-depth analysis techniques are required, capable of capturing the "global" changes within the cells. Recent advances in molecular biology, computer science and bioinformatics, allowed the generation of new in-depth techniques, also known as the "omics" techniques. They are defined as investigating large amount of data, representing the structure and function of an entire compositions of a biological system, at a particular level [128]. The four "big omics" techniques include genomics, proteomics, metabolomics, and transcriptomics, where transcriptomics, for example, allow the detection and analysis of all transcripts (mRNAs) expressed in a biological system at a given time [129]. Microarrays and RNA-Seq are currently the state-of-the-art techniques to perform transcriptome analysis. The difference is in general, that microarray techniques evaluate thousands of defined transcripts at the same time, including almost all expressed genes in a biological sample [130], whereas RNA-Seq is an un-targeted approach, evaluating all transcripts present at the time. In subsequent exposure studies, we used the microarray technique, as it is more cost-effective and faster than RNA-Seq, although the resolution is lower and it can have unspecific cross-reactions, leading to a higher background

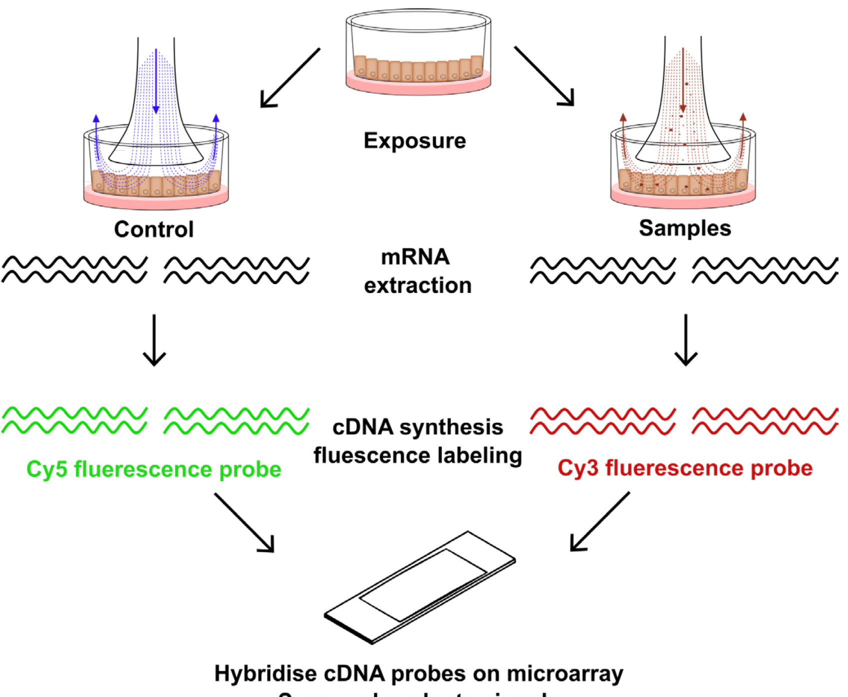


Figure 12: Basics of gene expression analysis by microarrays. Cells are cultivated in the same way and exposed according to the experimental design. Following exposure, cells are harvested, lysed and mRNA is isolated and purified. In the next step cDNA (complementary DNA) is reverse transcribed and differently fluorescently labeled, dependent on experimental control or sample of interest. Afterward the control as well as the sample cDNA probes are hybridized on the microarray to the corresponding primer before detection, quantification, and differential evaluation of the fluorescence.

signal. **Figure 11** depicts the general steps for a microarray-based transcriptome analysis and starts with the cultivation as well as the treatment of the cells. In the steps, the cells get harvested, the mRNA is extracted, reverse transcribed into complementary DNA (cDNA) and differentially labeled dependent on the treatment (Cy5 for the control, CY3 for the sample). For the quantification of the labeled fragments, they get hybridized on a microarray chip and are scanned for the signal intensity. As mentioned above, the location of the complementary target strand is known, allowing to allocate each signal to a target gene, while comparing directly the control to the sample. Beside the microarray analysis, we used for differential gene expression analysis also the quantitative real-time-PCR (QRT-PCR) approach, which is similar, but much more targeted and simpler than the microarray technique [131]. For QRT-PCR the mRNA is extracted, reverse transcribed into cDNA and amplified by PCR with specific labeled oligonucleotide primers for specific genes of interest. During PCR, after each amplification step, the intensity is measured, allowing the quantification of the gene of interest. For the final evaluation, the detected intensity is normalized to housekeeping genes as well as different experimental and technical controls.

4. Results and Discussion

4.1 Evaluating the toxicological impact of anthropogenic air pollutant properties by specific single source exposures

Sensitization to airborne allergens is the main risk factor for allergic respiratory diseases and the prevalence has been increasing globally in the last few decades [20-22]. The amount of airborne allergens, like birch pollen or pollen from birch homologous groups, increased in the past few decades in Europe [132] and was directly correlated to an increased sensitization to birch pollen in northern Sweden [133]. Beside direct allergen exposure, anthropogenic air pollutants had been identified as a major risk factor for the development of various diseases [16, 68, 93, 101]. Epidemiological studies found significant correlations to respiratory and cardiovascular disease [134, 135] but which particle properties, including size, mass, shape, surface structure or chemical composition induces these effects, is still not known [136-138]. To obtain insights into these factors, we performed single exposure studies at the ALI to specific generated aerosols like secondary organic aerosols (SOA). SOA are dominant components of atmospheric PM, which are formed though the oxidation of VOCs from biogenic and anthropogenic sources [139]. Comparing atmospheric aged combustions soot particles of gaseous biogenic (β-pinene) and anthropogenic (naphthalene) precursors, we could observe in both mono- (A459, BEAS-2B) and co-culture (A549 + EA.hy926) greater toxicity-related effects compared to primary soot particles (SP) [104, 140]. Especially SOA_{Nap} induced an NRF2 oxidative stress response, additional redox signaling and inflammatory signaling compared to $SOA_{\beta Pin}$. Chemical characterization revealed that $SOA_{\beta Pin}$ contained mostly aliphatic compounds from β-pinene photooxidations whereas SOA_{Nap} contained a significant fraction of oxygenated aromatic products which are probably the cause of greater toxicity related effects [140]. Interestingly, photochemical aging of nano-sized soot cores increased their organic carbon fraction and oxidation state, changing their effect on human lung cells. SP induced only mild toxicity, but photochemically aged SP increased DNA damage, oxidative damage, NRF2-mediated oxidative stress, induced augmented cytokine secretion and an AhR response activation. The differences are possibly caused by surface modifications, which lead to different molecular pathway activation [141]. To further confirm the impact of organic loads, we compared in another study aerosols with similar elemental carbon cores and physical properties but different organic loads. Our results indicated, similar than before, that with higher organic content (highOC), there was an increased induction of the xenobiotic metabolism, which is likely due to present PAHs, like benzo-a-pyrene, in the highOC aerosol [142]. Another single exposure study where we specifically targeted a specific source was regarding new construction materials containing carbon fibers. We evaluated the release of $PM_{2.5}$ during dry cutting of carbon composite concrete (C_3), thermal treated carbon composite concrete (tC_3) and carbon rods (CR) and could show that all materials induced in both mono- and co-cultures inflammatory cytokine release, cytotoxicity and genotoxicity [143]. Especially dry cutting of CR induced strong inflammatory responses which were shown to be due to high levels of

produced bisphenol A during the cutting process. Overall, all these studies indicated the usefulness of targeted single exposure ALI studies and the importance to combine them with comprehensive physicochemical analysis to connect the observed cellular effects to the various aerosol properties. Airborne pollutants can have, dependent on their physical and chemical properties, various adverse effects on human lung cells and are thus involved in various respiratory, but also cardiovascular and gastrointestinal diseases [16, 93, 144, 145].

4.2 Airborne compounds and their potential involvement in adjuvant- and protective-effects on respiratory allergic diseases

In recent studies, there are indications that airborne compounds are among the major influencing factors on allergic disease onset [17], but not always in a detrimental way. Dependent on the environment, there are compounds which increase (adjuvant effect) or decrease (protective effect) the effect of allergens. In this work, we evaluated these effects in *in vitro* cell model systems exposed with different state of the art ALI exposure techniques and used comprehensive toxicological as well as chemical analytical techniques for evaluation.

4.2.1 Evaluation of potential adjuvant effects

The most common respiratory allergies are triggered by pollen or house dust mites and the resulting allergic effect is increased with additional exposure to combustion aerosols derived from fossil fuels. In several epidemiological studies exacerbation of allergic diseases was related to anthropogenic combustion aerosols, indicating an adjuvant effect [146-149]. To study allergic adjuvant effects, exposure of submerged *in vitro* cell cultures has been used in the most studies, but in recent years there is a shift to more realistic air liquid interface exposures (ALI) [109]. Using ALI exposure techniques has the big advantage that the various systems are adapted to different kinds of aerosols, allowing for subsequent exposures in a more realistic way and hence the possibility to observe mechanistical synergistic or antagonistic cellular effects. In this regard, we performed two studies, where the first was a biogenic pre-exposure followed by an anthropogenic exposure, and the second, a study where we started with an anthropogenic exposure, followed by a biogenic exposure: In the first part,

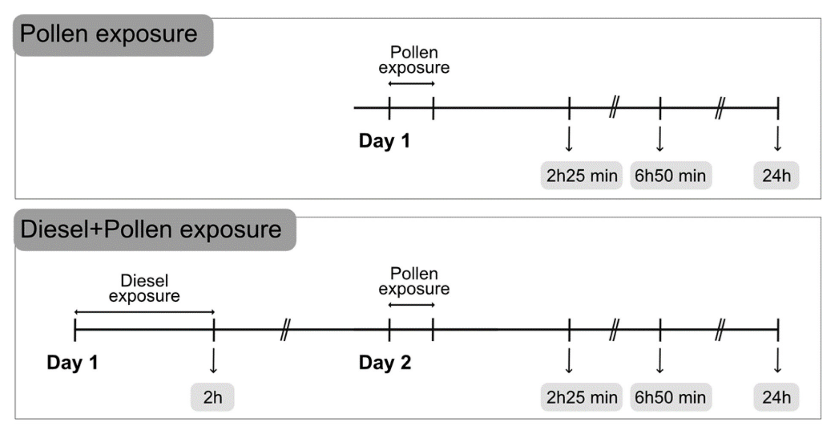


Figure 13: Experimental Setup of diesel exhaust exposure and whole birch pollen exposure.

subsequent ALI interface exposures were applied to investigate the effect of allergen pre-exposure on subsequent UPF exposure. For the first exposure, the Vitrocell® cloud chamber was adapted and utilized, which works on the principle of nebulization of the tested birch pollen extract (BPE) or house dust mite extract (HDME) in a chamber and the sedimentation of the generated droplets on the cells. 4h or 24h after first exposure of the human bronchial epithelial cells (BEAS2B), the second exposure to UFP was performed in AES from Vitrocell® for 2h. The UFP aerosol was generated through propane burning in a miniature combustion aerosol standard (miniCAST) generator and the gas phase as well as particle phase was directly guided to the exposed cells. The used aerosols used for exposure was physicochemical characterized with online and offline techniques. To gain insights into adjuvant effects and the underlying basic cellular mechanisms, following both exposures and an additional 2h of post exposure, toxicity, genotoxicity, and in-depth transcriptional changes were evaluated.

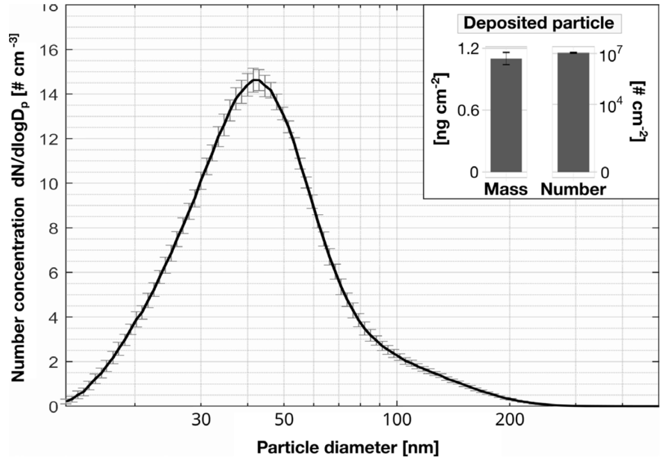


Figure 14: UFP size distribution and estimated particle/mass deposition on the cells

In the second part, subsequent exposures were performed to evaluate if diesel exhaust exposure has an adjuvant effect on subsequently whole birch pollen exposed human bronchial epithelial lung (BEAS-2B) cells. In this context, the cells were first exposed in the AES to diesel exhaust for 2h generated with a diesel CAST, following the next day the 4mg pollen exposure in the specially designed pollen-ALI [150]. The biological endpoints were 2h 25min, 6h 50min or 24h after the pollen exposure. To evaluate the effect of diesel exhaust exposure on the cells, controls were treated identical but were exposed to clean air (CA) instead of diesel exhaust (**Figure 13**).

4.2.1.1 Physicochemical aerosol characterization of combustion aerosols

Scanning mobility particle sizer (SMPS) measurements of UFP recorded a geometric mean diameter of 40.1 ± 1.4 with a mean particle concentration of $1.0 \times 10^6 \pm 0.6 \times 10^6$ cm^{-3}, which was confirmed by the condensation particle counter (CPC) showing a similar concentration of $1.1 \times 10^6 \pm 0.4 \times 10^6$ cm^{-3} (**Figure 14**) and thus within the commonly used size range definition of UFP [134]. In comparison to previous studies the UFP particle concentration was higher compared to ambient rural (0.0026×10^6 cm^{-3}), roadside (0.048×10^6 cm^{-3}) [151] or specific work environments ($0.08 – 0.35 \times 10^6$ cm^{-3}) [152], but less than what was used in comparable experimental settings with 163×10^6 cm^{-3} [153]. In the context of cell exposures, it is of important to constantly monitor the amount of generate particles but even more so to evaluate the deposited dose during the exposure. The estimated deposited dose of particles on the cells was

1.1 ± 0.06 ng cm^{-2}, which is approximately 10-fold higher compared to ambient human exposure in the tracheobronchial region over 24 h [154]. This deposition estimation was calculated with the multiple-path particle dosimetry (MPPD) model and resulted in 0.10 – 0.13 ng cm^{-2} for UFP in humans over 24 h. Nevertheless, this is only an approximation as several factors including aerosol characteristics, age and gender directly influencing the deposited dose. A comprehensive chemical characterization of the generated UFP indicated an aerosol rich in organic compounds (60.7 ± 6.1 % of total carbon), where within the 30 most abundant compounds identified, 12 of the 16 "*high risk PAHs*" as defined by the IARC were present including possible and probable confirmed carcinogenic compounds (**Table 1**) [66]. As summarized in **Table 2** had the generated diesel exhaust a larger geometric mean than the produced UFP with 136 ± 8 nm, a lower particle concentrations of 240 ± 59 µg m^{-3} and almost no organic content with a black carbon concentration of 207 ± 50 µg m^{-3}. Thus, the physical parameters are comparable to other human chamber exposures [155, 156]. Similar to the generated UFP, comprehensive chemical characterization showed the production of various monocyclic- and polycyclic- aromatic hydrocarbons, such as acenaphthylene, fluoranthene and fluorene, similar to a previous diesel exhaust study [157], although not as many "high risk PAHs" as were detected in the UFP with high organic content. Moreover, several oxygenated species were identified, like 1,2-Cyclopentanedione or Butylated Hydroxytoluene but also 2,6-ditert-butyl-4-nitrophenol, a known stabilization agent for diesel fuel.

Table 1: 15 most abundant identified compounds within the generated aerosol

Name	Structure	Toxicity as per IARC
Dibenz(a,h)anthracene		2A
Cyclopenta[cd]pyrene		2A
Pyrene		3
Fluoranthene		3
Chrysene		2B
Benzo[ghi]perylene		3
Phenanthrene		3
Cyclopenta[cd]fluoranthene		NA
Benzo[k]fluoranthene		2B
Pyrene, 1-methyl-		NA
Benzo[a]pyrene-4,5-dione		NA
9H-Cyclopenta[a]pyrene		NA
Benzo[a]pyrene		1
4H-Cyclopenta[def]phenanthrene		NA
Fluoranthene, 4,5-dihydro		NA

4.2.1.2 Toxicological assessment

Our study showed that BPE, HDME and UFP caused genotoxic effects in bronchial epithelial BEAS-2B cells (**Figure 15**). While HDME has already been reported to induce DNA strand breaks [158], this is the first time that BPE is reported to directly induce genotoxic damage in *in vitro* bronchial epithelial cells. Chan, Tan [158] previously evaluated ROS production and DNA damage from aeroallergens in BEAS-2B cells under submerged conditions and, while they demonstrated a strong dose-dependent genotoxic effect of HDME, they observed no DNA damage with ragweed pollen extract (RWE). The difference could be caused by their experimental setup as they used submerged exposures, compared to our ALI exposure including both the particle- and gas-phase. In a previous study, intranasal application of RWE in mice causes pollen induced oxidative DNA damage via reactive species [159]. Thus, a possible explanation could be that cultured BEAS-2B cells at ALI behave more similarly to *in vivo* settings and are therefore more sensitive to pollen extracts compared to *in vitro* cells exposed under submerged conditions.

Table 2: Diesel exhaust (CAST) exposure characteristics. Mean and s.d. are given. N ≥ 3 for all time points.

Parameters	Mean (± s.d.)
Mean particle size (nm)	135.6 ± 7.8
Particle number (#/cm3)	46 301 ± 5424
Total PM (µg/m3)	239.6 ± 59.0
BC (µg/m3)	207.5 ± 49.9
Total Carbon (µg C/m3)	130.5 ± 5.9
OC/EC ratio	0.01 ± 0.01

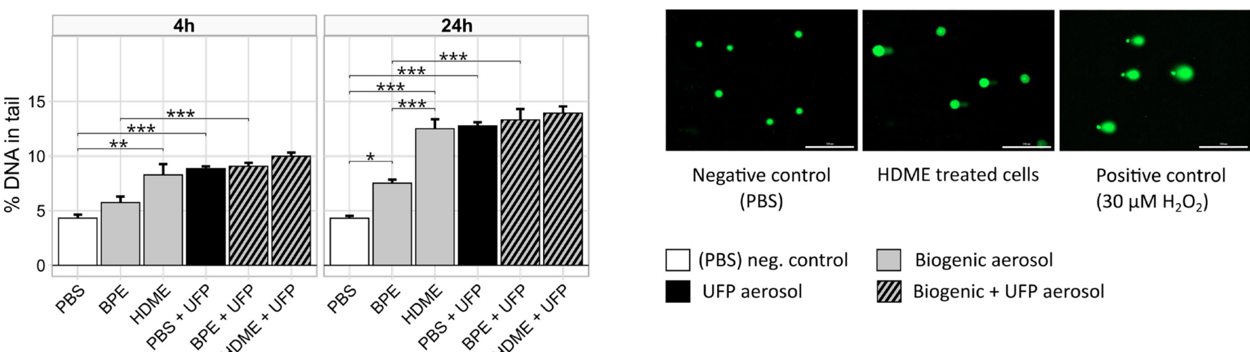


Figure 15: Induced genotoxicity in BEAS-2B cells exposed at air-liquid interface to biogenic aerosol, UFP aerosols or sequentially to both aerosols and representative micrographs resulting from comet assay (scale bar = 200 µm). Genotoxicity is shown as mean percentage of DNA in tail during comet assay. Results are presented as mean ± standard errors of the mean (SEM) of n = 3. ANOVA with Tukey's multiple comparison as post hoc test was used for statistical comparison to negative control. *p<0.05, **p<0.01, ***p<0.001.

While our results showed that single exposures to BPE, HDME and UFP cause DNA damage to BEAS-2B cells, subsequent exposure of allergens and UFP did not increase DNA damage in an adjuvant manner. UFP exposure significantly increased DNA damage of BPE pre-treated cells, though this increase seemed to reach a threshold at 12-15 % In addition, UFP exposure of HDME pre-treated

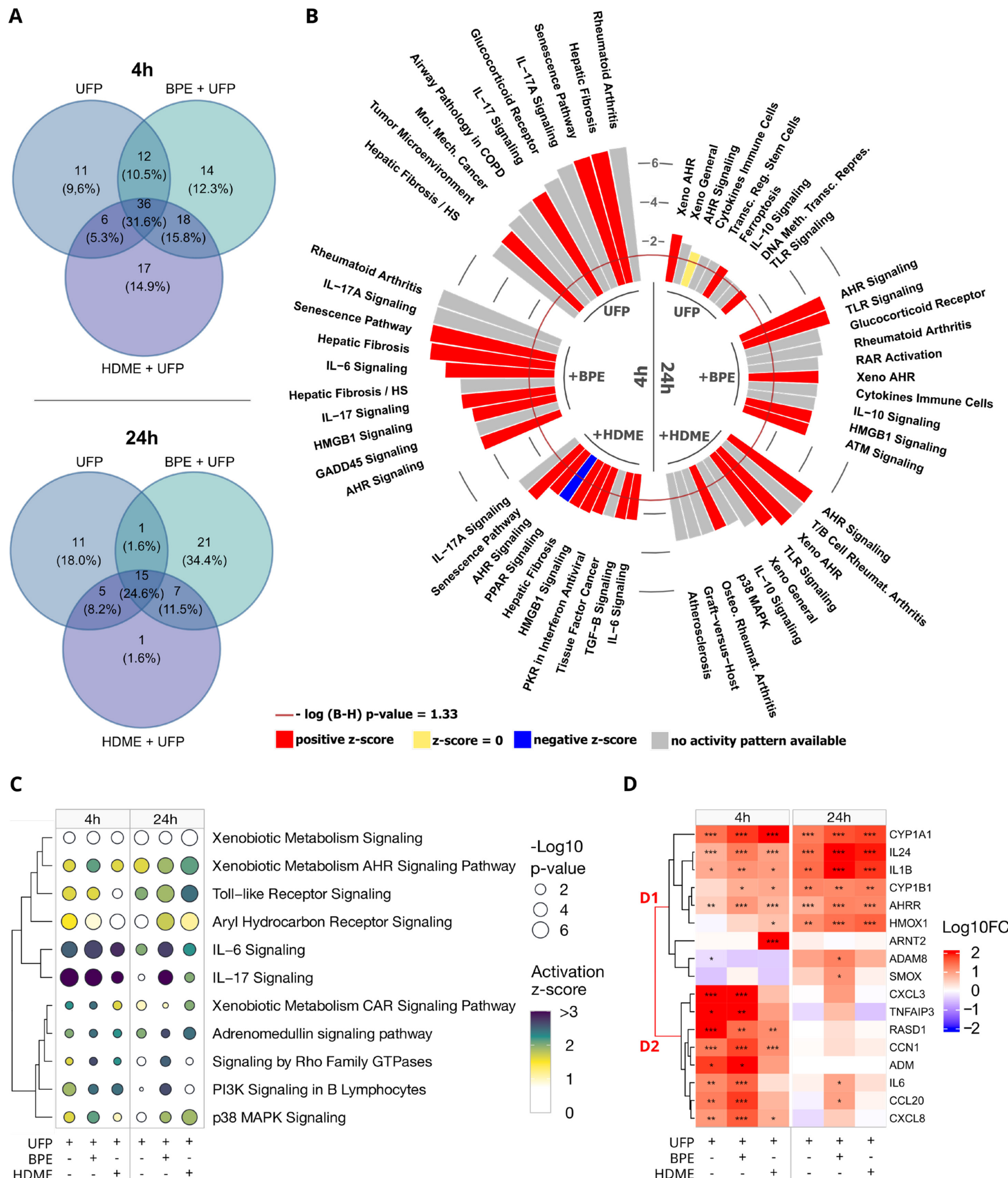


Figure 16: Transcriptomic analysis of BEAS-2B cells exposed to UFP, BPE + UFP or HDME + UFP. (**A**) Venn diagrams of all differential regulated genes after 4 or 24 h exposure to biogenic aerosols followed by UFP aerosol exposure. (**B**) 10 most different regulated canonical pathways compared to negative (PBS and clean air exposed) control. Values are Benjamini Hochberg (H-B) corrected – log p-values with a threshold of 1.33. Color indicates z-score. (**C**) Canonical pathways related to

allergic and xenobiotic responses identified by Ingenuity Pathway Analysis (IPA). (**D**) Heatmap of significant and selected relevant genes related to allergic and xenobiotic responses. n = 1 for UFP, BPE + UFP and HDME + UFP condition; n =2 for negative controls. Identically treated cells exposed to PBS and clean synthetic air served as reference for the analysis.

cells showed a similar level of DNA damage induced by only UFP or HDME treated cells. The amount of inducible genotoxic effects with our biogenic- and UFP-exposure seemed to be limited. A possible explanation could be associated with AhR signaling, as transcriptomic analysis indicated in both aerosols the involvement of AhR binding ligands. This agrees with previous observations where PAH- and HDME-induced DNA damage is associated with AhR binding [160, 161]. This could indicate that PAHs contributed to the observed genotoxic damage after UFP exposure as has been shown in a previous study of Bonetta et al. [162], where higher PAH concentrations was associated with higher DNA damage. Moreover, in a recent study of Juarez Facio et al. (2022) organic UFP containing PAHs induced an increase in oxidative stress, which can lead to DNA damage and like our findings they did not observe an increase of cytotoxicity.

In-depth transcriptomic analysis indicated that the cellular response changed from pro-inflammatory related pathways and DEGs after short allergen pre-incubation (4 h) to AhR mediated xenobiotic related pathways after longer pre-incubation (24 h). The evaluation of the most differentially expressed canonical pathways indicated that UFP exposure, independently of the biogenic pre-treatment, caused an upregulation of IL-17(A) signaling and senescence pathways after 4 h pre-treatment but not after 24 h pre-treatment. Pro-inflammatory IL-17 and IL-17A signaling pathways are related to allergic diseases, and has been linked to the pathogenesis of diverse autoimmune inflammatory diseases [163]. UFP aerosol exposure after 24 h pre-incubation to allergens was differentially regulating a more specific xenobiotic AhR metabolism response compared to 4 h pre-treatment. This indicated that xenobiotic signaling was likely involved in our observed DNA damage like previous findings where urban particulate matter induces DNA damage pathways in BEAS-2B cells [164]. Juarez Facio et al. (2022) made similar observations, where organic UFP exposure of BEAS-2B cells was increasing the upregulation of xenobiotic related genes, while decreasing inflammatory marker genes. IL-10 signaling was differentially regulated and has been shown to have a regulatory function in allergic rhinitis and asthma and participates in suppression of cellular, allergy related Th2 responses in favor of a Th1-type response [165, 166]. Hence, we could observe that the indicated canonical pathways changed depending on the time of pre-incubation but reflected the connection to the observed genotoxicity. The allergen pre-treatments indicated a change of the most differentially expressed pathways to more allergy-inflammatory (IL-6-, HMGB1-, TLR-signaling) and xenobiotic (AhR signaling) related pathways. IL-6 signaling and HMGB1 signaling are involved in a wide variety of stimuli caused by tissue damage, stress and human (lung-) diseases [167-169] . Moreover, HDME has already been shown to activate HMGB1 with subsequent inflammatory cytokine production [170-172]. We observed a possible enhanced upregulation of AhR signaling in cells pre-treated with biogenic aerosols at both time points. Previous studies reported similar findings for HDME showing

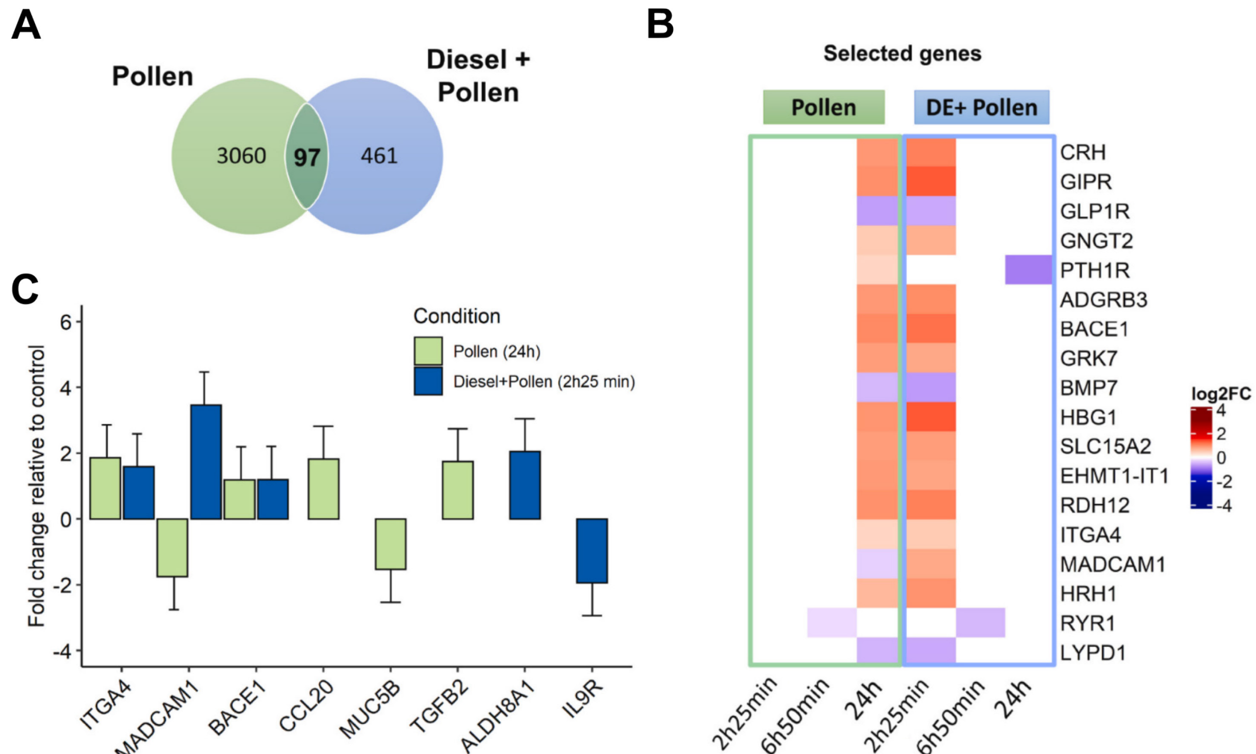


Figure 17: Comparison between differential expressed genes of BEAS-2B cells exposed to pollen alone or with preceding diesel exhaust exposure. (A) Venn diagram summarizing the total number of differential expressed genes in both pollen exposure alone and combined exposure. (B) Heatmap of selected significant expressed common genes related to immune and allergic responses (18 of all 97 expressed genes found with both exposure conditions). (C) mRNA expression of select genes by QRT-PCR that were expressed in transcriptome data, for pollen alone and/or combined exposure.

facilitation of pro-inflammatory cytokine release through AhR in mice [161] and human bronchial epithelial cells [173, 174]. Interestingly, in another study a possible adjuvant effect could be observed on BEAS-2B cells, where cells pre-treated to diesel exhaust reacted faster to birch pollen than without diesel exhaust pre-exposure [175]. We found a similar adjuvant effect as AhR signaling was one of the top canonical pathways with allergen pre-treatment after 4 h alone and was strongly pronounced after 24 h with and without allergen pre-treatment.

Comparable to the subsequent biogenic- and UFP-exposure there were no difference with or without prior diesel exhaust exposure coupled to native pollen exposure in cytotoxicity (below 15%) and the metabolic activity (above 85%) indicating low exposure doses, that preserves cell viability. But transcriptomic analysis revealed that BEAS-2B cells with pre-exposure to diesel exhaust reacted faster to birch pollen than compared to cells exposed only to birch pollen. This was seen as the highest differential regulation of genes was shifted to 2h 25min compared to 24h in case of only pollen exposure. Comparing the differentially expressed genes between the two different treatments, only pollen exposure affected much more genes (3060 genes) compared to subsequently exposed cells (461 genes) (**Figure 16 A**),

indicating a diesel-mediated numbness, that was also observed in previous studies [176, 177]. A total of 97 differentially regulated genes were found by both treatments. **Figure 17 B** shows a subset of genes related to relevant immune responses. **Figure 17 C** illustrates genes from qPCR results, confirming the transcriptome data. Significantly up-regulated genes after subsequent exposure are related to response to extracellular stimulus, cell adhesion, detoxification and organic hydroxy compound metabolism, whereas inhibited genes related to processes of cellular response to nitrogen and organic cyclic compounds. Most of the differentially regulated genes were up regulated in both settings, and diesel pre-exposure enhanced their expression. Interestingly, most of the upregulated genes, like ITGA4 or MADCAM1, are known to be induced in asthma and chronic inflammation [178, 179]. GnGT2, BACE1 and FBP1 were found in other studies to be correlated to asthma [180-182]. Down-regulated genes after subsequent exposure related to the regulation of immune response and th2 differentiation, like IL9R, which is particular important for asthma [183]. Overall, comparing pollen alone with subsequent diesel and pollen exposure, pathways related to inflammation were expressed earlier in diesel-exhaust pre-exposed cells and most of those genes, like ITGA4, SLC15A2, HRH1, GNGT2 and BACE1, were previously associated with allergic asthma and pro-inflammatory responses [178, 180, 182]. Thus, our results show that fresh diesel exhaust pre-exposure has an adjuvant allergic effect that could not only result in earlier and stronger allergic reactions with the enhancement of inflammatory related pathways, but also suggest that diesel exhaust disables the epithelial barrier. In case of the biogenic pre-exposure before UFP exposure, the pre-treatment also changed the cellular response, but in another direction, leading to increased xenobiotic- and inflammatory related pathways. This difference is probably caused by many factors, including the different physical properties as well as the difference in organic carbon content, including many PAHs. Overall, this illustrates that subsequent ALI exposures provide a faster way to generate evidence for adjuvant allergic effects and can help to increase the knowledge regarding the underlying cellular mechanisms involved in the onset of allergic diseases.

4.2.2 Evaluation of potential protective effects

As illustrated above, various sources or environments can have an adjuvant effect on allergic diseases but on the other side, there are environments which are able to protect from the onset of allergic diseases. Several epidemiological studies have shown that growing up on a traditional cow farm can reduce the risk of allergic diseases onset [75, 82, 184]. Factors like raw milk consumption, the presence of cows and the presence of straw are important [75], indicating that the accompanying microbial richness (and endotoxin), triggering the immune system, lead to the observed protective effect [80, 81]. The focus on previous studies was mainly on bigger compounds like endotoxin, proteins, and bacterial species [80, 81], although there are much more compounds which needs to be evaluated. Hence, the mechanisms of this effect are still unknown. Evaluating the allergic protective effect of a whole environment, requires standardized and reproducible sampling of both particle- and gas-phase, a comprehensive

physicochemical analysis and subsequent *in-vitro* cell exposures. Correlating the biological response from fast and reproducible submerged *in vitro* cell exposures to sampled fractions and/or identified compounds provides a powerful tool to evaluate complex environments.

In this study, we applied a sophisticated sampling techniques coupled to *in vitro* cell exposures and focused on the small organic compounds regarding the protective effect of farm environments. As cow sheds induce this effect but not sheep sheds [75], we sampled the inhalable fraction ($PM_{2.5}$ and gas phase) in two cow sheds and two sheep sheds and compared them with each other. Following tailored sampling, the samples from a known protective cow shed were split into two and *in vitro* BEAS-2B cells were exposed to the total fraction or the < 3 kDa to evaluate immune modulatory effects. At the same time the cow and sheep sheds were chemically characterized with a non-targeted IDTD method coupled to GC×GC-ToFMS and further evaluated with conducted ANOVA-PCA to identify specific SVOCs for cow sheds. After the analysis, *in vitro* cell exposures were performed with all sheds as well as differently identified compounds evaluating the differentially expressed genes of relevant genes.

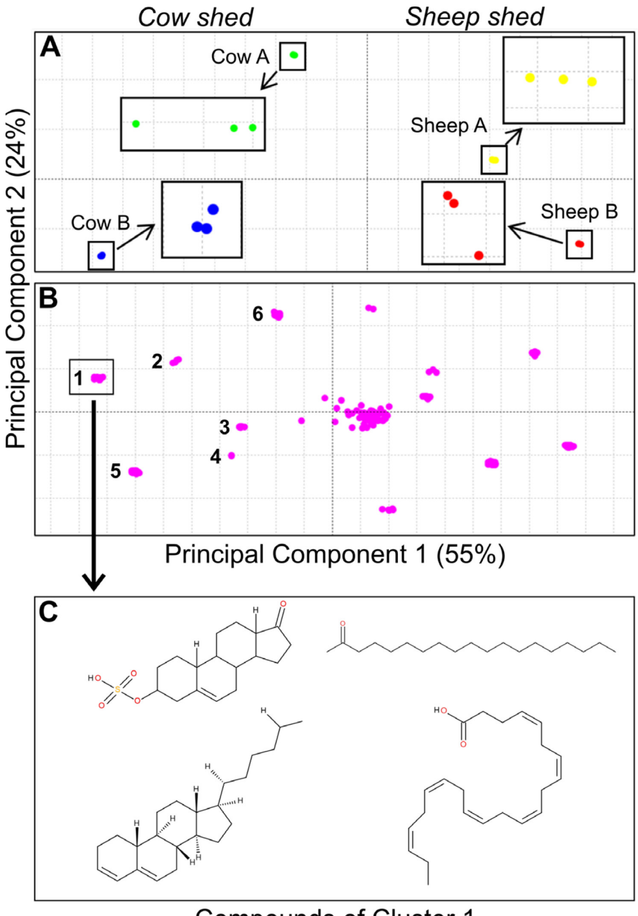


Figure 18 Scores plot of ANOVA-PCA with identified compounds. A: Scores plot (p = 0.1 %) of the four different stables. B: Corresponding loadings plot with important clusters (1-6) regarding cow stables. C: 4 identified compounds of cluster 1

4.2.2.1 Chemical aerosol characterization

In total, we were able to detect approximately 700 SVOCs to be present on the collected PM. Following ANOVA PCA, the different shed types were separated along principal component 1 (PC1) with 55% and the individual sheds along PC2 with 24% as illustrated in the loading plot of **Figure 18**. With this plot specific patterns of SVOC composition could be identified and the most relevant loadings for cow sheds are displayed in the loading plot in clusters labeled 1-6 (**Figure 18 B**). As the cluster 1 illustrates the most distinct relevance for both cow sheds, it was investigated in more detail and allowed to identify four chemical compounds (**Figure 18 C**): 2-nonadecanone, dehydroisoandrosterone sulfate (DHEA-S), docosaheanoic acid (DHA) and cholesta-3,5-diene. To allow for correct identification of these key

compounds, they were quantified again with corresponding standards. This resulted in the conformation of DHEA-S (12 ng m^{-3}) and could confirm the compound classes of the other three compounds. In previous studies, two of the four identified compounds were already corelated to protective cellular functions in human cells (Table 3). The omega-3 fatty acids (DHA) were identified as milk constituent in mammalians and are correlated To an allergic protective effect through anti-inflammatory properties [185]. Similar, DHEA-S are the most abundant circulating steroid hormones in humans and are currently discussed as the "superhormone" with "anti-aging" properties [186].Secretion of DHEA from the adrenal cortex has an age related decline and it is associated with many age-related changes, like immunosenescence or atherosclerosis, but also with an possible allergy protective effect [186, 187].

4.2.2.2 Toxicological Assessment

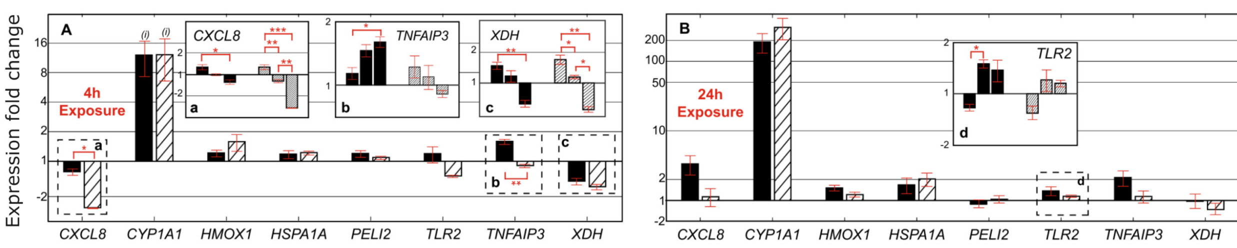


Figure 19: Differential gene expression analysis of BEAS-2B cells exposed to total cow shed extract (CS-AT) or < 3 kDa fraction (CS-AS) after 4 h and 24 h. A: 4 h exposed cells. B: 24 h exposed cells. Small squares indicate respective genes following exposure to three (15.6 µg mL-1, 125 µg mL-1, 500 µg mL-1) different concentrations of CS-AT and CS-AS. *p<0.05, **p<0.01, ***p<0.001.

To get a first indication if the small fraction (< 3 kDa) has a different immune modulatory response with selected genes compared to the total fraction, qPCR analysis was performed with exposed BEAS-2B cells (**Figure 19**). Several genes were significantly differently regulated with the two different treatments, comprising genes related to allergic inflammation (*TNFAIP3*) [80], allergic regulation (*XDH*) [188, 189] and general inflammation (*CXCL8*) after 4 h exposure as well as another inflammatory related gene (*TLR2*) [190] following 24 h exposure. These results indicate that the immunomodulatory effects observed are caused by the < 3 kDa fraction as similar observations were made with the total fraction. Identifying the smaller fraction as relevant, the next step was to compare the protective and reportedly not protective sheds with each other (**Figure 20**). The observed regulation was for most genes similar but PELI2 and TLR2 were differentially regulated after 4h exposure indicating a distinct regulation for cow shed. PELI2 has been indicated to regulate signaling from TLRs and IL-8 over IL-1 induction [191] and is therefore related to a general inflammatory response. Moreover, PELI2 has been linked to the signaling of TLRs [192] and might thus, directly regulate the distinct TLR2 regulation from our study. TLRs belong the first line of defense, by initiating the innate immune system, corresponding to the ligand. In this regard, it was suggested that the activation of TLRs by microbial products exerts a suppressive effect against allergic diseases [193]. HMOX1 was increased after 4 h and significantly up-regulated after 24 h but only in the cow shed B. HMOX1 is an oxidative stress marker gene, involved in the protection against chemical

reactive species [194], and is probably up-regulated by level of PAHs in the cow shed B. We could identify significantly higher level of PAHs in the cow shed B compared to the other sheds, comprising eleven of the targeted PAHs.

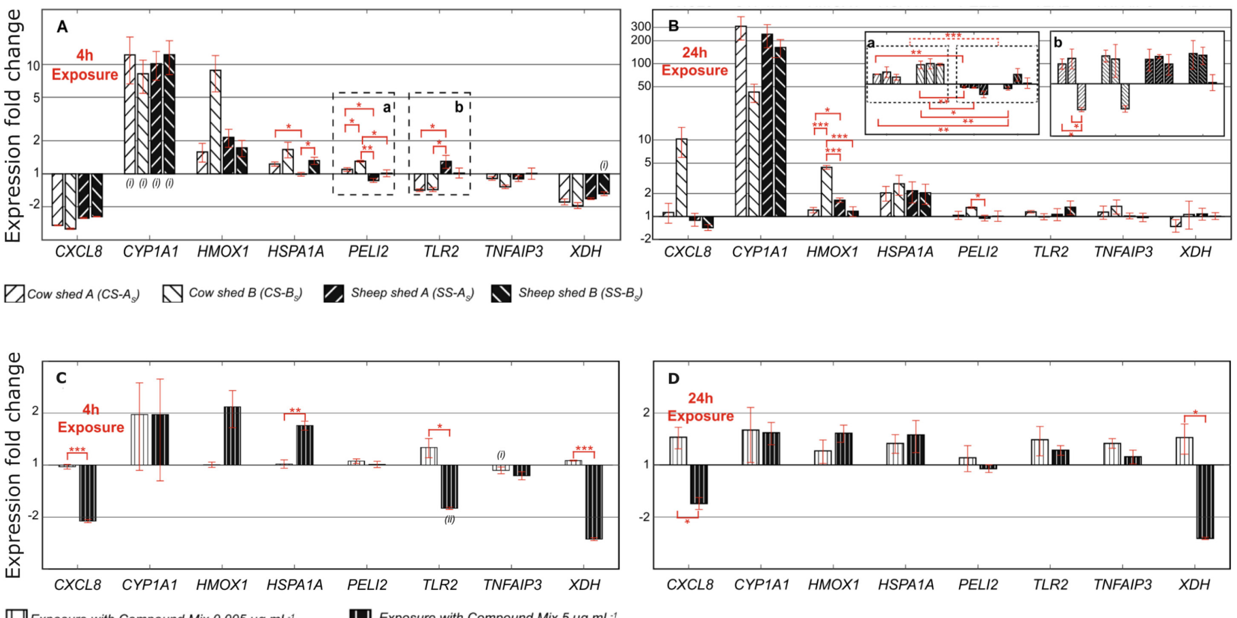


Figure 20: A,B: Differential gene expression analysis of BEAS-2B cells exposed < 3 kDa fraction of cow shed extract or sheep shed extract. C,D: Gene expression analysis of cells exposed to 4 identified compounds of cow shed. A,C: 4 h exposed cells. B,D: 24 h exposed cells. Small squares indicate PELI2 (a) or TLR2 (b) following exposure to three (15.6 µg mL^{-1}, 125 µg mL^{-1}, 500 µg mL^{-1}) different shed concentrations. *p<0.05, **p<0.01, ***p<0.001

Observing distinct immune modulations by the small fraction as well as with the cow sheds, we continued by exposing the cells to an artificial made mixture, made from the identified key components in cow sheds. Regulation of *HMOX1*, *HSPA1A*, *XDH*, *TNFAIP3* and *CXCL8* approach the pattern of the cells exposed to the small fraction (**Figure 20**). *CYP1A1* was much less pronounced than the shed extract exposed cells, illustrating the impact of PAHs on the *CYP1A1* regulation as was already shown previously for exposed *in vitro* A549 and BEAS-2B cells [195, 196]. *PELI2* regulation was not differentially regulated but TLR2 after 4h indicated similar regulations compared to the small fractions from both cow sheds (**Figure 20 A**). Thus, *TLR2* regulation is an interesting target to further investigate the mechanism that might contribute to the allergy protective effect of farming environments. Overall, the combination of sophisticated sampling and analyzation methods with in *vitro cell* exposures enabled us for the first time to identify four key components from protective cow shed environments and showed the involvement of small organic compounds in inflammatory-, oxidative stress-, and xenobiotic related pathways, indicating an involvement in the investigated protective effect.

4.3 Enabling long term ambient air *in vitro* exposure studies

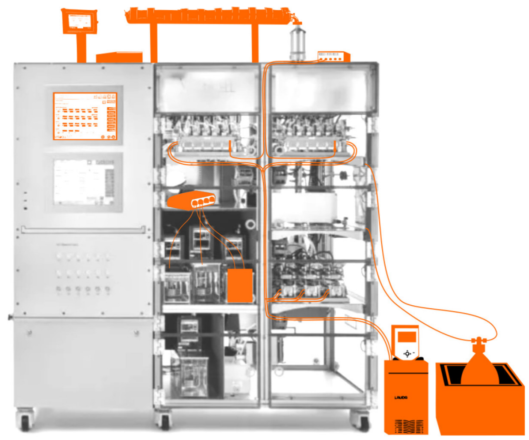


Figure 21: AES with long-term adaptations in orange.

In summary, we got some relevant indications for allergy- adjuvant and allergy protective effects by using various state of the art ALI exposure systems coupled to toxicological and analytical chemical procedures. But the observed effects were small (though significant) and showing only acute biological effects. This results in a need for research and development, of which some has already been addressed. The cell culture model system needs to be improved. There is the need for a real sensitization assay for epithelial human lung cells, reflecting realistic epithelial barrier integrity conditions [110]. Additionally, there is the need for long-term ALI exposures to directly evaluate realistic environmental exposure condition. By combining both, a primed model system for sensitization with realistic long-term environmental air exposures, will help to gain further insights into the sensitization process, the mechanisms involved as well as potentially help to identify compounds leading to allergic sensitization. Within this work, the long-term exposure need has been addressed and an adapted system has been validated.

In recent years, *in vitro* model systems gained increasing interest in respiratory research due to ethical concerns and high costs of animal use. There has been extensive research on possible *in vitro* alternatives, reaching the point of using ALI-models (cells grown at the ALI) with reproducible ALI exposures systems exposing to both particle- and gas-phase. These systems have already been successfully applied to evaluate tobacco whole-smoke (TWS) or various engine exhausts [175, 197, 198]. One of the major drawbacks of these exposure systems is regarding a relevant deposited dose. Using unrealistic large amounts of exposure material, to mimic a longer timeframe, is technically challenging to achieve, challenging to monitor and can possibly lead to increased formation of particle agglomerations through increased particle interactions. Using realistic low doses for exposure have the benefit of easier production and monitoring, but may result in no observable cellular response, due to low deposition rates, short exposure durations and the use of cell models. In a study of Herzog, Loza [199] et al., the effect of silver nanoparticles was evaluated in an acute dose and lifetime dose through *in vitro* ALI exposures. Their results showed cytotoxic and pro-inflammatory effects only with the highest dose and by applying acute endpoints, as is common practice for cell cultures [199]. Therefore, to be able to study chronic inhalation scenarios *in vitro*, prolonged realistic low-dose applications are required.

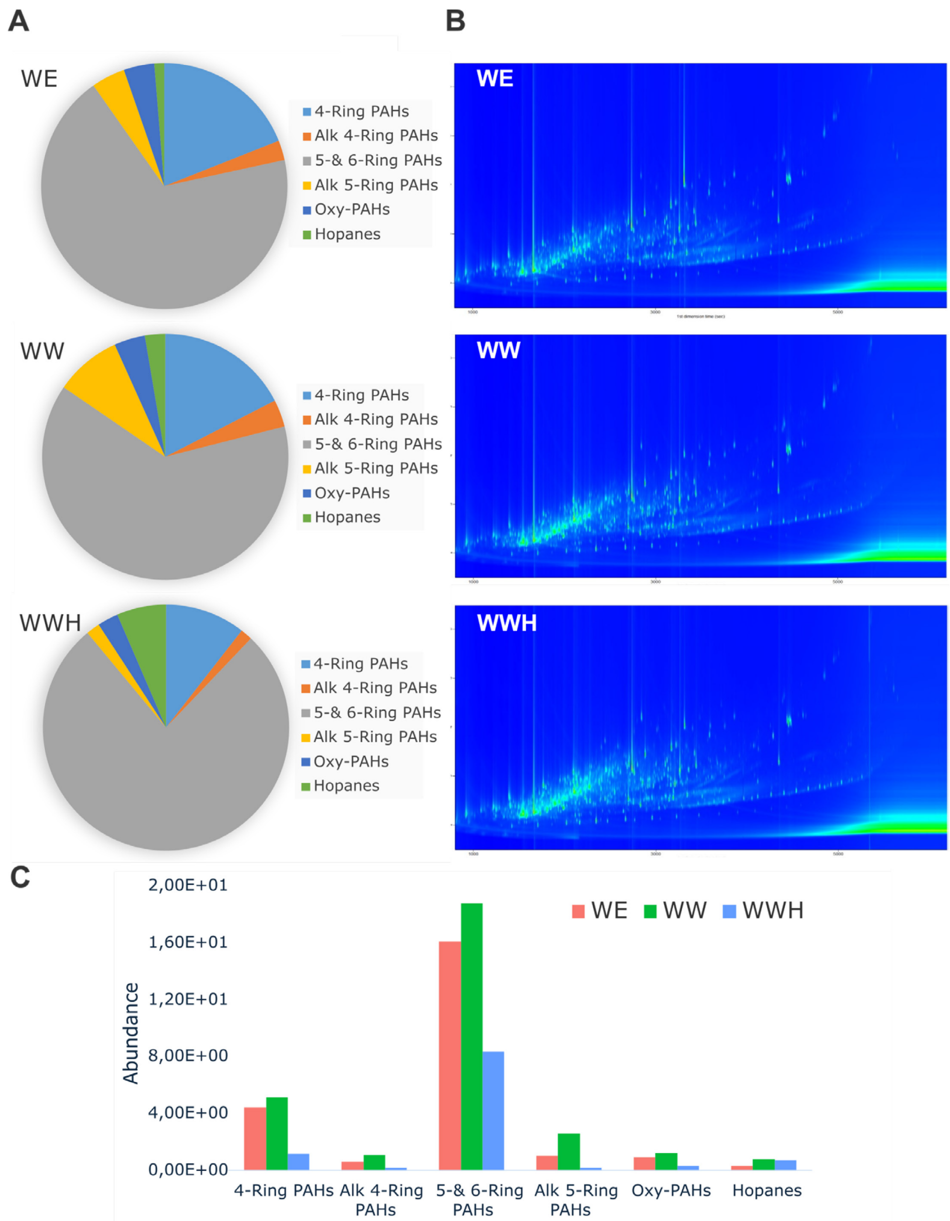


Figure 22: (A) Abundance/Area (normalized to d12-Benz(e)pyrene) of different compound classes with potential health related effects for the three different samples. Alk – alkylated; Oxy – oxygenated. (B) Pie-charts of the area percentage of each class within each

In this study, an optimized AES for long term in vitro exposures was validated and for the first time Calu-3 cells were continuously exposed to outdoor ambient air for 72h. The Calu-3 cells were exposed to ambient air either during the weekend (WW), the work week (WE) or the work week with a holiday on the second exposure day (WWH). Toxicological endpoints were assessed following the 72h exposures, and the physicochemical properties of the aerosol was characterized using online and offline techniques. To our knowledge, we are proposing for the first time an approach enabling long-term exposures of *in vitro* lung cells to continuous flow exposures. As mentioned before, previous studies with similar systems from

our and other groups usually used single exposure durations of 2-4 h [104, 143, 175, 198, 200, 201] or repeated exposures of 4h or 12h per day [111, 202]. The longer exposure duration allowed to reduce the test compound dose to realistic concentration or even use directly ambient air on human lung cells [202].

4.3.1.1 Physicochemical aerosol characterization

Physical characterization of the ambient air showed a common air pollution composition for central Europe, including CO, NO, NO_2, O_3, $PM_{2.5}$.and PM_{10}. We observed lower mass and particle number concentrations (PNC) during WE, compared to WW and WWH which is to be expected due to the closed companies and reduced traffic. Moreover, during the occasional rain fall, mass concentrations, PNC and $PM_{2.5}$ were strongly decreased due to wet deposition [203]. The concentration of $PM_{2.5}$ during our exposures range from 7 µg m^{-3} to 11.6 µg m^{-3} and was thus always below the legal German limit [204]. Continuing with un-targeted GC×GC-TOFMS we could detect similar compound groups in all exposures in various concentrations (**Figure 22**). 5- & 6- ring PAHs and 4-ring PAHs were the most abundant compound groups. Alkylated 5-Ring PAHs were most present during the WW with 9 % with much less concentrations during the WE and WWH (respectively 4 % and 2 %). Interestingly, the biggest fraction of hopanes (7%) we could detect during the WWH. Hopanes are specific organic markers, found in the lubricating oils used by both gasoline- and diesel powered motor vehicles [205] and we would expect more traffic during WW. Performed abundance estimates as well as targeted GC-TOFMS showed similar results. Within the different timeframes we could observe similar compound classes with various concentrations. Particularly during the WW, we measured higher PAH concentration as well a shift towards alkylated 4- and 5-ring PAHs. Recently Moradi, Hung [206] showed that some alk-PAHs had a huge impact on the toxicity of urban air, but if our observed biological effects are due to the increase of alk-PAHs or the general increase of PAHs in WW is not clear.

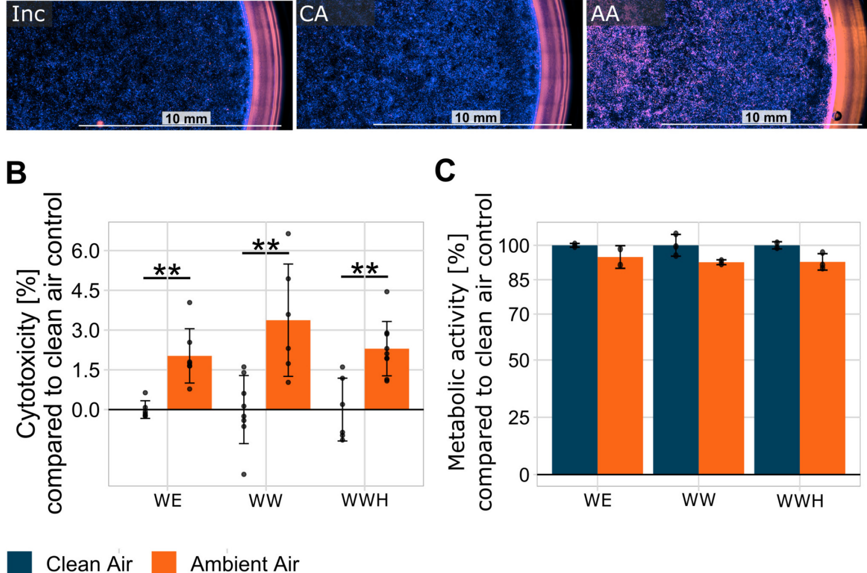


Figure 23: (**A**) Representative live/dead cell. (**B**) Percentage cytotoxicity measured by LDH assay and normalized to the total LDH release. (**C**) Metabolic activity measured by resazurin assay and compared to the Inc.

4.3.1.2 Toxicological Assessment

In our study, ambient air exposure for 72 hours caused significant increase in cytotoxicity in bronchial epithelial cells in all exposure periods, and decrease in metabolic activity (**Figure 22 A,B**), but no epithelial integrity loss or pro-inflammatory cytokine release. In previous studies and reviews, *in vitro* cytotoxicity has been attributed to compounds including PAHs, phthalates, different UFPs or gases [90, 105, 153, 207, 208]. Compared to our study, focused the above mentioned studies on single sources and concentrations which were set much higher than real concentrations to represent much longer periods of time. Interestingly, studies investigating the combined effect of two different compounds or sources reported attenuated cellular responses [60, 175, 200] and increased toxicity even at very low doses [16, 209]. This could explain the cytotoxicity we observed following our exposures. Even though we had realistic low dose concentrations, due to the complex mixture and longer exposure time to ambient air, the aerosol induced an effect in the robust Calu-3 cell line. Interestingly, while we could not observe difference in IL-6 or IL-8 release, neither *CXCL8* gene expression following AA exposure, there was a more pronounced down regulation of IL-6 gene expression indicated in WE and WW compared to CA exposed cells (**Figure 24**). Gene expression studies on Calu-3 cells are scarce, but previous studies working with bronchial epithelial lung cells BEAS-2B reported a down regulation of *CXCL8* and, similar to our study, *IL6* following UFP or particulate matter emission exposure at the ALI or submerged [153, 210]. *IL6* downregulation is probably connected and mediated by PAHs, indicating the possible link between PAHs and the negative regulation of inflammatory response. This could explain our observations, where indicated downregulation of *IL6* correlates to increasing PAH concentrations. On the other side, we observed an indication for upregulation of the proinflammatory marker *IL1β* in cells exposed to AA in all timeframes and the xenobiotic marker gene *CYP1B1* following AA exposure during WE and WW (**Figure 24).** Considering these results, it is important to regard the timeframe as well as antioxidant and DNA

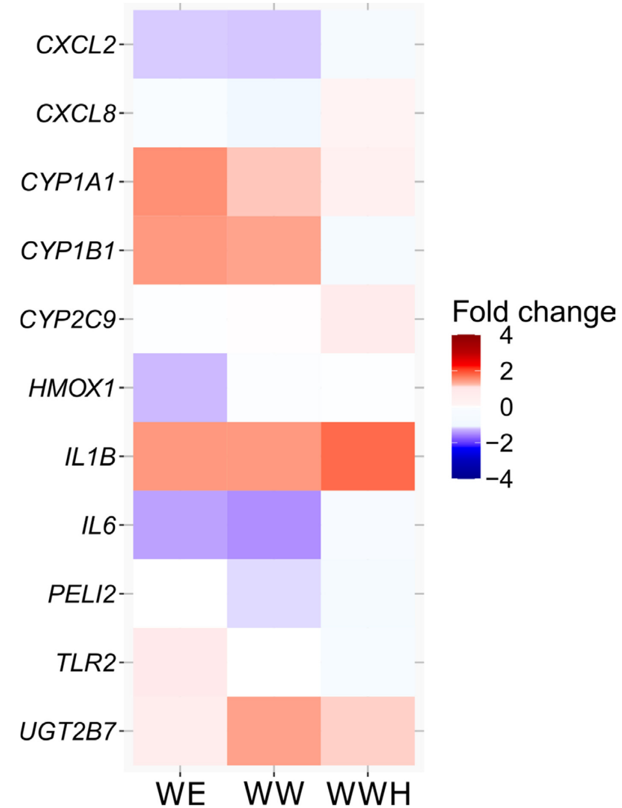


Figure 24: Gene expression fold change of inflammatory (CXCL2, *CXCL8*, *IL1B*, *IL6*, *PELI2; TLR2*, *UGT2B7*) and Xenobiotic (*CYP1A1, CYP1B1, CYP2C9, HMOX1*) related marker genes. Expression fold change is normalized to two housekeeping genes (*GAPDH*, *RFLP13A*) and compared to clean air control. WE: weekend, WW: work week, WWH: work week with holiday on second day. Data are shown as mean (n >= 2)

repair capabilities of the cell model [211]. Due to the long duration of the exposure, various repair mechanisms could be initiated, dealing with the acute response, and possibly leading to an underestimation of the results. Overall, with this study we could validate that the optimized AES System is able to continuously expose in vitro cells for 72 h and that calu-3 cells are a possible option, especially as we did not observe any differences between CA exposed cells and Inc controls. To our knowledge, for the first time were in vitro cells exposed continuously to ambient air, resulting even in small biological responses. We could thus propose a feasible approach to gain insights into various pressing topics, ranging from real dose workplace safety evaluations to possible underlying mechanisms of allergic diseases.

5. Conclusion and Outlook

In this dissertation, we demonstrated the importance of using comprehensive physical and chemical characterization combined with different state of the art *in vitro* exposure systems to evaluate allergy adjuvant- and allergy protective-environments. A special focus was on using adapted ALI exposure systems to specific sources or test compounds, which were used for single or sequential exposures, evaluating very complex environments or research questions. With these techniques, we successfully performed sequential exposures to either allergen extracts and lab-generated organic UFP or native birch pollen and diesel exhaust, to evaluate potential adjuvant effects in a pulmonary *in vitro* cell model. In both studies, we could observe immune-modulatory effects following sequential exposures. Pre-exposure to nebulized BPE increased allergy-pro-inflammatory canonical pathways, while xenobiotic-related pathways were increased by BPE and HDME pre-exposure, indicating a common mode of action. With BPE and HDME pre-exposures, AhR signaling activation was promoted, highlighting the potential role of AhR signaling in response to airborne pollutants and associated allergic disease development. Indeed, chemical characterization of the generated organic UFP aerosol revealed the presence of several high-risk PAHs, known for their induction of AhR signaling. Moreover, we could demonstrate that both biogenic pre-exposures and organic UFP exposure caused genotoxicity, although, subsequent exposures did not increase genotoxicity. Pre-exposure of BEAS-2B cells to diesel exhaust and then to native birch pollen resulted in stronger allergic reactions with the enhancement of inflammation related pathways. Interestingly, by comparing to native pollen only exposed cells, we could demonstrate that diesel exhaust pre-exposed cells show a shift to a faster inflammatory response when pollen is encountered. Especially genes related to severe asthma and inflammation (BACE1, GNGT2, ITGA4 and MADCAM1) were expressed earlier. Thus, our results show in all performed subsequent exposures, immune-modulatory effects in an adjuvant manner. This emphasizes the importance of considering synergistic effects of various aerosols during the evaluation of potential allergy adjuvant effect during the onset of allergic diseases. To evaluate the involvement of small organic compounds in the allergy protective effect of indoor cow shed environments, we used fast and efficient submerged *in vitro* exposures combined with tailored sampling and analysis techniques. The comprehensive chemical analysis of cow- and sheep sheds allowed us to

discriminate between both farming environments and enabled the identification of four key components found only in cow sheds. QPCR analysis revealed distinct differential gene expression patterns between the small organic fraction and the total fraction, indicating an important involvement of the small organic compounds in various immune-modulating processes. Moreover, we could demonstrate that exposures to the small fraction of cow- and sheep-sheds result in similar regulation patterns with a few distinct differences. General stress related gene *PELI2* and allergic inflammation related gene *TLR2* were significantly up regulated, indicating their possible involvement in the allergy protective effect. Interestingly, exposure to an artificial mixture containing the identified key components from cow sheds resulted in a comparable regulation pattern of *TLR2* and thus their possible involvement in the observed immune-modulating effects. Within this dissertation, we demonstrated several applications of state-of-the-art *in vitro* ALI exposure systems and their importance in studying air pollutants and their involvement in various diseases, though limited to acute cellular responses. Thus, to extend the scope of application in the direction of chronic cellular responses, we validated an AES optimized for continues long-term *in vitro* exposures and performed the first continues 72 h outdoor ambient air exposure of a pulmonary *in vitro* cell model at ALI. Our results showed that reproducible and continues long-term exposures for 72 h are feasible, without impairment of the ALI cell layer. This allowed direct exposure of lung epithelial cells to outdoor ambient air without changing the physicochemical properties of the aerosol. Following ambient air exposure, our results show a significant increase in cytotoxicity, independent of the exposure period, with a reduction of metabolic activity. Although chemical analysis revealed varying PAH concentration, including high-risk PAHs, this was not reflected in cytotoxicity. We could also observe that the concentration of PAHs in the aerosol seems to correlate with the downregulation of the inflammatory related gene *IL6*. In summary, the system can be implemented to study the effects of different indoor and outdoor environments without changing the aerosol. Combining it with an advanced cell model system that could be kept at an ALI for an even longer period of, might enable the study of pathophysiological mechanisms associated with air pollutants.

Future studies could improve the quality and range of application of *in vitro* exposures at ALI by using *in vitro* coculture systems that include different physiologically relevant cell lines. Multicellular ALI-models increase the range of possible outcomes and are comparable to *in vivo* conditions as they have realistic cell-cell communications. Furthermore, noninvasive biological online measurement techniques could further expand the spectrum of endpoints, especially during prolonged long-term exposures. Regarding the allergy adjuvant- and allergy protective-effect, further studies should be conducted evaluating different important environments or sources, as indicated by several epidemiological studies, and provide more insights of the underlying mechanisms on the onset off allergic diseases.

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7. Appendix

7.1 Lists of Abbreviations

AA	ambient air
AES	automated exposure station
AhR	aryl hydrocarbon receptor
ALI	air-liquid interphase
ANOVA	analysis of variance
AOP	adverse outcome pathway
BACE1	beta-secretase 1
BaP	beno[a]pyrene
BC	basophil cell
BPE	birch pollen extract
C_3	carbon composite concrete
CA	clean air
CAST	combustion aerosol standard
cDC	convetional dendritic cells
cDNA	complementary DNA
CO	carbon monooxide
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
CPC	condensation particle counter
CR	carbon rods
CXCL	chemokine (C-X-C motif) ligand
Cyp	cytochrom P450
DAPI	4`,6-diamidino-2-phenylindole
DC	dendritic cell
DEG	differential expressed gene
DEP	diesel exhaust particles
DHA	docosaheanoic acid
DHEA-S	dehydroisoandrosterone sulfate
DMEM/F12	high-glucose Gibco Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12
DNHP	2,4-dinitrophenylhydrazine
ELISA	enzyme-based immunosorbent assay
FBS	fetal bovine serum
GC	gas chromatography
GM-CSF	granulocyte-macrophage colony stimulating factor

GnGT2	guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-T2
GSH	glutathione
HDME	house dust mite extract
HMGB1	High-Mobility-Group-Protein B1
HMOX1	heme oxygenase 1
HRH1	histamine H1 receptor
HSPA1A	heat shock 70 kDa protein 1
IDTD	in-situ derivatization thermal desorption
IgE	immunoglobulin E
IL	interleukin
IPA	ingenuity pathway analysis
ITGA4	integrin alpha 4
LC	lymphoid cell
LC	liquid chromatography
LDH	lactate dehydrogenase
MC	mast cell
MDA	malondialdehyde
MPPD	multiple-path particle dosimetry
mRNA	messenger RNA
MS	mass spectrometry
NADH	nicotinamide adenine dinucleotide
NO	nitric oxide
NO_2	nitrite
NRF2	nuclear factor erythroid 2-related factor 2
O_3	ozone
OA	organic aerosol
OC	organic content
OH	hydroxyl radicals
P/S	penicillin and streptomycin
PAH	polycyclic aromatic hydrocarbons
PCA	principle component analysis
PELI2	protein pellino homolog 2
PM	particulate matter
PM2.5	PM with upper size limits of 2.5 µm
POA	primary organic aerosols
QFF	quartz fiber filter
qPCR	quantitative real-time-PCR
rH	relative humidity

RNASeq	RNA sequencing
ROS	reactive oxygen species
RWE	ragweed pollen extract
SLC15A2	solute carrier family 15 (H+/peptide transporter), member 2
SO_2	sulfur dioxide
SOA	secondary organic aerosols
SP	soot particle
SVOC	semi volitalie organic carbons
T	temperature
TEER	transepithelial electrical resistance
TEM	transmission electron microscopy
TH cell	T-helper cell
TLR2	toll-like receptor 2
TNFAIP3	tumor necrosis factor alpha-induced protein 3
TSLP	thymic stromal lymphopoietin
TWS	tobacco whole-smoke
UFP	ultra fine particles
VOC	volatile organic compounds
WE	weekend
WHO	word health organization
WW	work week
WWH	work week with holiday
XDH	xanthine dehydrogenase

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7.4 List of Publications

Zimmermann, Elias Josef, et al. "Biological impact of sequential exposures to allergens and ultrafine particle-rich combustion aerosol on human bronchial epithelial BEAS-2B cells at the air liquid interface." *Journal of Applied Toxicology* 43.8 (2023): 1225-1241.

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https://doi.org/10.1016/j.scitotenv.2024.175727

Currently unpublished Manuscripts:

Zimmermann, Elias Josef, et al., " Toxicological effects of long-term continuous exposure to ambient air on human bronchial epithelial Calu-3 cells exposed at the air-liquid interface." (manuscript submitted to Environmental Research)

Offer, Svenja, et al., " The Chemical Composition of Secondary Organic Aerosols Regulates Transcriptomic and Metabolomic Signaling in an Epithelial-Endothelial In Vitro Coculture." (manuscript submitted to Particle and Fibre Toxicology)

7.5 Contribution to conferences

Zimmermann E. J., Candeias J., Bisig C., Gawlitta N., Binder S., Pantzke J., Offer S., Huber A., Buters J., Di Bucchianico S., Oeder S., Zimmermann R. (2022). " Impact of sequential exposures to allergens and high organic ultrafine particles on human bronchial epithelial BEAS-2B cells at the air liquid interface." nternational Aerosol Conference in Athens. 4-9 September. Conference Talk.

7.6 Curriculum Vitae

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